No. 15-1413

United States Court of Appeals
for the Federal Circuit

THE BOARD OF TRUSTEES OF THE LELAND STANFORD JUNIOR UNIVERSITY,
Appellant,

v.

ARIOSA DIAGNOSTICS, INC.,
Appellee.

Appeal from the United States Patent and Trademark Office,
Patent Trial and Appeal Board, Case No. IPR2013-00308.

OPENING BRIEF OF APPELLANT

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May 4, 2015

CERTIFICATE OF INTEREST

I, R. Danny Huntington, counsel for Appellant The Board of Trustees of the Leland Stanford Junior University, certify the following:

1. The full name of the party represented by me is The Board of Trustees of the Leland Stanford Junior University.

2. The Board of Trustees of the Leland Stanford Junior University is the real party in interest.

3. There are no parent corporations or publicly held companies that own 10 percent or more of the stock of this party.

4. The law firms and the partners and associates that appeared for this party in the *Inter Partes* Review before the Patent Trial and Appeal Board or are expected to appear in this Court are:

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STATEMENT REGARDING ORAL ARGUMENT

The Board of Trustees of the Leland Stanford Junior University ("Stanford") requests oral argument.

STATEMENT OF RELATED CASES

This case is related to Civil Action No. 3:12-cv-05501 brought in the Northern District for California. That case involves Stanford and Ariosa Diagnostics, Inc. ("Ariosa"), which are both parties to this appeal. It also involves Verinata Health, Inc. ("Verinata"), which is a licensee of the patent involved in this appeal. There are two patents involved in that case, one of which is the patent at issue in this appeal – U.S. Patent No. 8,296,076. The district court case has been stayed pending this appeal.

The other patent at issue in the district court litigation is U.S. Patent No. 8,318,430 ("the '430 patent"). The '430 patent was involved in two *Inter Partes* Review ("IPR") proceedings – IPR2013-00276 and IPR2013-00277. Ariosa has appealed the Final Written Decision issued in those proceedings. *See Ariosa Diagnostics v. Verinata Health, Inc.,* No. 2015-1226 (Fed. Cir. filed December 29, 2014); *Ariosa Diagnostics v. Verinata Health, Inc.,* No. 2015-1215 (Fed. Cir. filed January 6, 2015).

The above-mentioned district court litigation has also been consolidated with another case in the Northern District of California between Illumina, Inc. and

Ariosa. *See Illumina, Inc. v. Ariosa Diagnostics, Inc.*, Case No. 14-cv-01921 (N.D. Cal. filed April 24, 2014). Currently pending before this Court is Illumina's appeal of the district court's denial of Illumina's motion to compel arbitration of Ariosa's breach of contract counterclaims in that action. *See Illumina, Inc. v. Ariosa Diagnostics, Inc.*, No. 2014-1815 (Fed. Cir. filed September 15, 2014).

JURISDICTIONAL STATEMENT

This Court has jurisdiction pursuant to 28 U.S.C. § 1295(a)(4)(A) of an appeal from a decision of the Patent Trial and Appeal Board ("PTAB") in an *Inter Partes* Review.

STATEMENT OF ISSUES

1. Whether the PTAB erred in its construction of the term "sequencing predefined subsequences" by not properly considering the plain language of the claims, the intrinsic evidence, the testimony of both parties' experts, and Ariosa's admissions in a parallel district court litigation.

2. Whether the PTAB erred in utilizing its erroneous construction of "sequencing predefined subsequences" to conclude that the claims of the '076 patent are invalid as anticipated under 35 U.S.C. § 102(e).

3. Whether the PTAB erred by failing to separately analyze claim 9's validity despite finding a different, narrower scope for claim 9 in the context of its claim differentiation analysis.

4. Whether the PTAB erred in utilizing its erroneous construction of "sequencing predefined subsequences" to conclude that the claims of the '076 patent are invalid as obvious under 35 U.S.C. § 103(a).

PRELIMINARY STATEMENT

This case has its origins in the district court, where Stanford sued Ariosa for infringement of the '076 patent. In the district court, Ariosa focused on the claim term "predefined," and argued that it restricted the '076 claims to a targeted sequencing approach. Stanford agreed with this. However, Ariosa's construction did not stop there. Instead, in an effort to avoid an infringement finding, Ariosa attempted to further narrow the claims such that they would only encompass a single method of targeted sequencing. However, the '076 disclosure is broader than that single method. The district court recognized this, and so refused to limit the claims as Ariosa wanted.

Faced with a claim construction that offered Ariosa no possibility of avoiding an infringement finding, Ariosa filed the IPR that is the subject of this appeal. However, Ariosa could not find prior art that matched the targeted sequencing approach claimed in the '076 patent. As a result, Ariosa had to flip-flop its claim construction position, and now argue the claims are ***not*** limited to targeted sequencing, but also encompass the opposite – random – sequencing approach.

However, the expert Ariosa hired to defend its changed position in the IPR could not do so, and when cross-examined quickly agreed that the '076 claims are directed to targeted sequencing. She also agreed that the Lo reference, which is alleged to anticipate the '076 claims, does not teach targeted sequencing.

Stanford's own expert in the IPR concurred with these admissions, as well as pointing out that the '076 specification discloses targeted sequencing. Importantly, Stanford's expert also emphasized that the specification specifically contrasts targeted sequencing with random sequencing.

Thus, the record before the PTAB contained (1) expert testimony that the claims are limited to targeted sequencing, (2) the limiting claim language itself, i.e., "sequencing ***predefined*** subsequences," (3) specific embodiments of targeted sequencing in the specification, (4) a direct contrast between targeted and random sequencing in the specification, and (5) Ariosa's admissions in the district court that the claims are limited to targeted sequencing. And yet, despite all of this evidence, the PTAB determined that the claims encompass random sequencing. In reaching this determination, the PTAB made no effort to reconcile its interpretation with the record before it.

Armed now with its overbroad construction, the PTAB concluded that the '076 claims are anticipated by the Lo reference. But, if the '076 claims are properly construed as limited to targeted sequencing (like the parties agreed in the

district court), then this determination cannot hold. The reason for this is simple - Lo does not teach targeted sequencing.

STATEMENT OF THE CASE

This case originates from Case No. IPR 2013-00308, instituted on November 20, 2013 to review claims 1 - 13 of U.S. Patent No. 8,296,076 ("the '076 patent"). JA000174-200. In instituting the IPR, the PTAB found the claims of the '076 patent are not limited to any particular sequencing technique. JA000181. In the PTAB's Final Written Decision ("the PTAB's Decision") that issued on November 19, 2014, the PTAB maintained its interpretation, and expressly stated that the term encompasses both random and targeted sequencing. JA000013. Based on that claim construction, the PTAB concluded that claims 1 – 13 of the '076 patent are invalid as anticipated or obvious in view of the cited prior art. JA000027-28. Stanford then filed a Notice of Appeal to this Court under 35 U.S.C. § 141 seeking review of the PTAB's Decision.

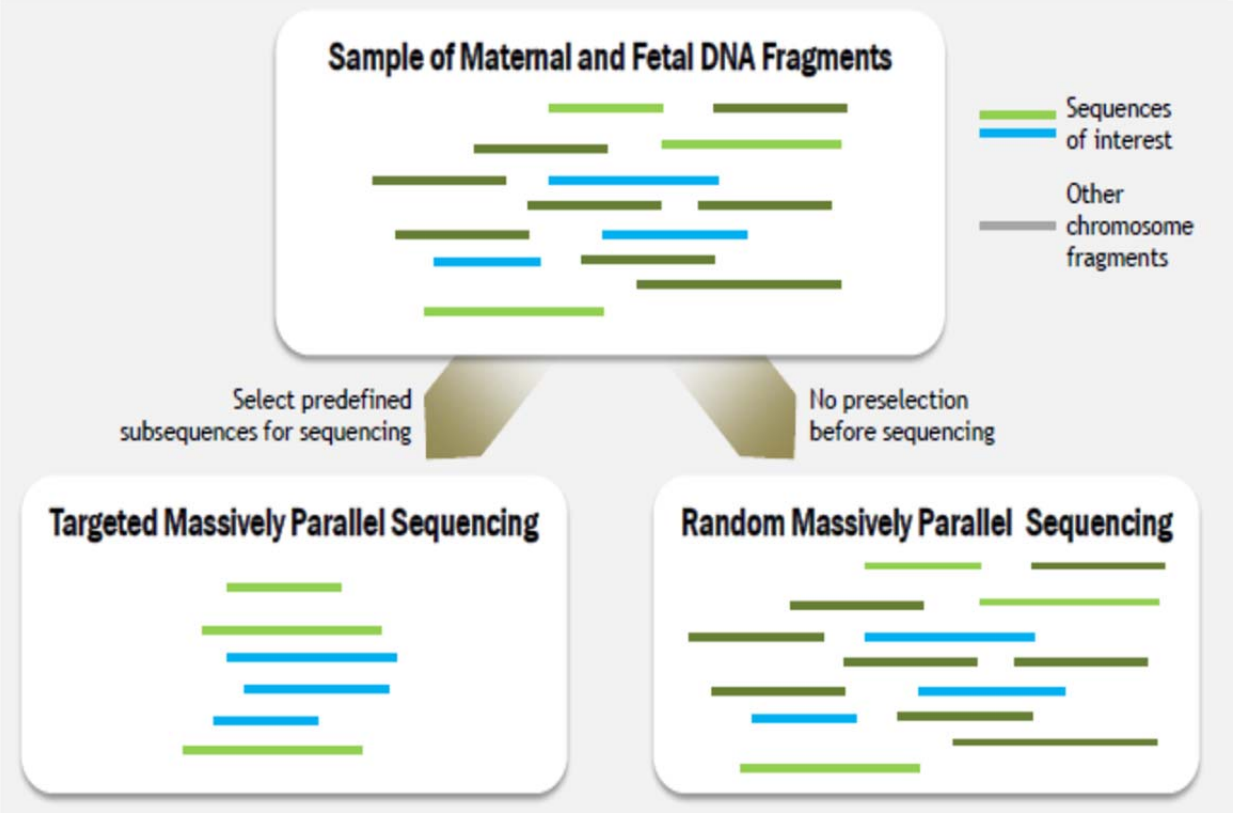
I. The Invention Claimed in the '076 Patent

The '076 patent is generally directed to non-invasive methods of detecting fetal aneuploidy, e.g., Down syndrome. JA000069. An essential part of the claimed method is the sequencing of maternal and fetal DNA. This is reflected in claim 1, the only independent claim challenged in the IPR:

> 1. A method of testing for an abnormal distribution of a chromosome in a sample comprising a mixture of maternal and fetal DNA, comprising the steps of:
>
> (a) obtaining maternal and fetal DNA from said sample;
>
> (b) sequencing predefined subsequences of the maternal and fetal DNA to obtain a plurality of sequence tags aligning to the predefined subsequences, wherein said sequence tags are of sufficient length to be assigned to a specific predefined subsequence, wherein the predefined subsequences are from a plurality of different chromosomes, and wherein said plurality of different chromosomes comprise at least one first chromosome suspected of having an abnormal distribution in said sample and at least one second chromosome presumed to be normally distributed in said sample;
>
> (c) assigning the plurality of sequence tags to their corresponding predetermined subsequences;
>
> (d) determining a number of sequence tags aligning to the predetermined subsequences of said first chromosome and a number of sequence tags to the predetermined subsequences of the second chromosome; and
>
> (e) comparing the numbers from step (d) to determine the presence or absence of an abnormal distribution of said first chromosome.

The ’076 patent specification describes two different approaches to sequencing. The first approach is “random” massively parallel sequencing, and the second approach is “targeted” sequencing, which can include the use of massively

parallel sequencing.[1] The difference between these two approaches is that the "targeted" sequencing approach utilizes a pre-selection step to select or "target" the DNA to be sequenced. JA003651. The pre-selection step ensures that only certain predefined sequences are molecularly sequenced. In other words, the pre-selection step is a physical selection step that excludes non-selected sequences from being molecularly sequenced. The difference between the two approaches can be illustrated as follows:



[1] Different terms of art are used throughout the IPR record to refer to the same sequencing technique. That is, the terms "targeted sequencing," and "directed sequencing" are synonymous, and refer to a sequencing technique that uses a pre-selection step. Similarly, the terms "random sequencing" and "shotgun sequencing" are synonymous, but they refer to a sequencing technique that ***does not*** use a pre-selection step.

JA000502.

As depicted above, "random" sequencing involves sequencing millions of randomly fragmented genomic DNA sequences. JA000072. Using this approach, the randomly fragmented DNA sequences within a given sample are randomly selected and sequenced. That is, there is no physical pre-selection step, and the specific identity (sequence), of the DNA is not known until ***after*** sequencing. JA001193; JA003650-51.

The "targeted" sequencing approach differs from the "random" approach because it contains a pre-selection step, i.e., the sequence of the DNA is known ***before*** the sequencing step. JA003650-51. Knowledge of a given sequence prior to the actual molecular sequencing step increases the sensitivity of the method because it enables one to focus only on certain regions (as opposed to entire chromosomes) within the human genome. JA000074 at 13:53-57. The '076 patent specifically contrasts the two approaches:

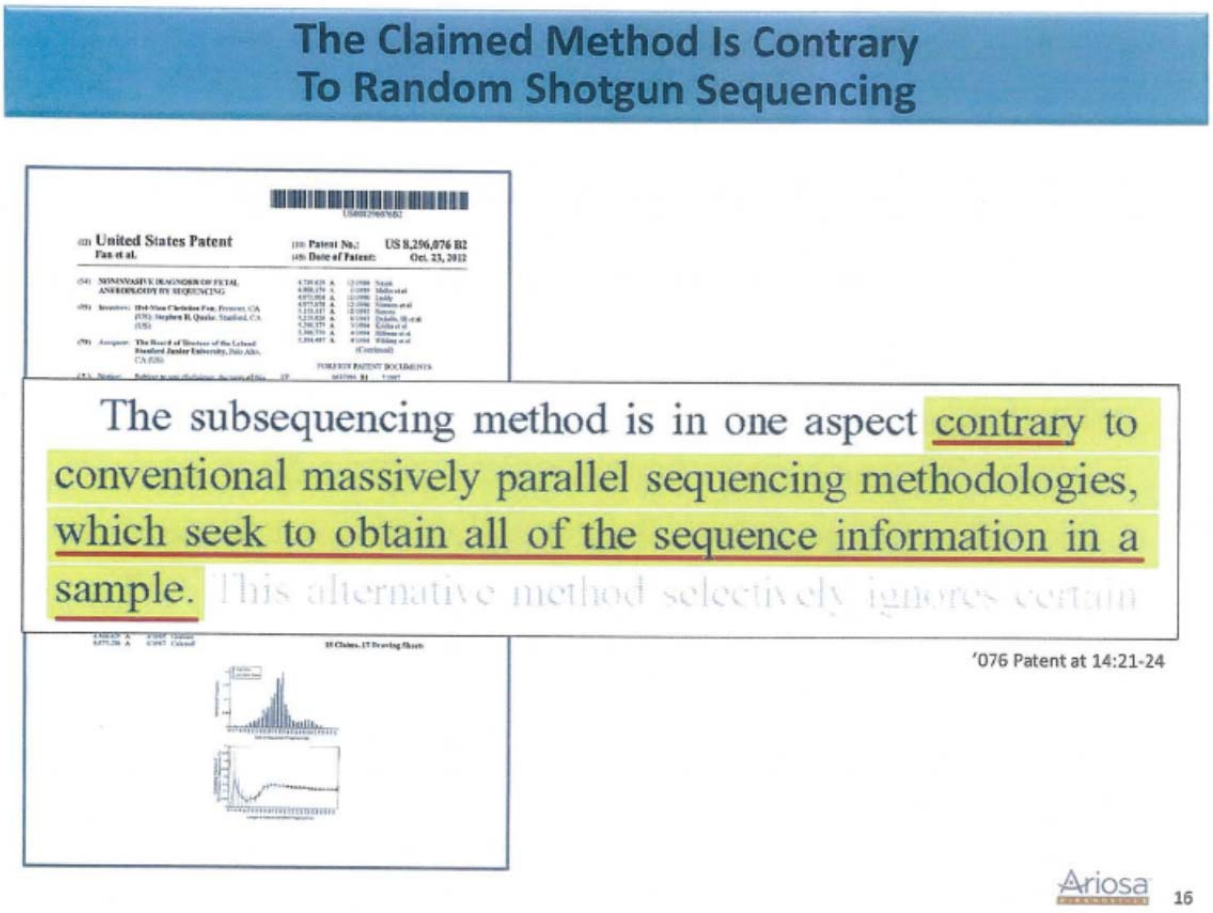
> The [targeted] subsequencing method is in one aspect contrary to conventional massively parallel sequencing methodologies, which seek to obtain all of the sequence information in a sample. This alternative method selectively ignores certain sequence information by using a sequencing method which selectively captures sample molecules containing certain pre-defined sequences.

Id. at 14:21-27.

The ’076 patent further explains that the “targeted” approach involves “sequencing methods which select a priori sequences which map to the chromosomes of interest…” *Id.* at 13:54-57. The ’076 patent also describes methods other than targeted massively parallel sequencing for “sequencing selected subsequences,” e.g., sequencing by array or capture beads with specific genomic sequences. *Id.* at 13:65-14:1.

II. In the District Court, Stanford and Ariosa Agreed the Claims of the ’076 Patent Are Directed to Targeted Sequencing

Stanford and Verinata have asserted the ’076 patent against Ariosa in district court. During claim construction, Ariosa contended that the ’076 patent claims are directed to targeted sequencing, where only predefined sequences are sequenced. In a demonstrative that it used at the *Markman* hearing, Ariosa specifically highlighted the claim term “sequencing predefined subsequences” that appears in claim 1, step (b). JA003397. Ariosa’s demonstratives are clear that “the claimed method [in the ’076 patent] is contrary to random shotgun sequencing:”



JA003400. Notably, Ariosa supported its contention that the '076 patent claims are "contrary to random shotgun sequencing" by reference to the disclosure in the '076 patent contrasting a "targeted" sequencing approach from a "random" sequencing approach. *Id.*

Ariosa's claim construction briefing also characterizes the '076 patent as claiming a "targeted approach" to sequencing. JA003802 ("the claims of the '076 patent are directed to a specific method for determining aneuploidy by quantifying DNA through ***the targeted sequencing of 'predefined subsequences'*** of DNA…"), ("Though the claims recite a ***targeted approach***…"), ("The ***targeted approach***

claimed in the ’076 patent…”) (emphasis added). During the *Markman* hearing, Ariosa’s counsel also made it clear that the ’076 claims are directed to a “targeted” approach:

> Now, let’s begin with the claim itself. The claim says you’re sequencing - - again, very important as far as the sequencing step. ***You’re sequencing what? Predefined subsequences. Again, this is a targeted approach.*** They’re subsequences. Second, they’re not just any subsequences. They’re predefined. Selected a priori.

JA004222-23 (emphasis added). At no point during the district court claim construction proceedings did Ariosa ever suggest that the claims of the ’076 patent encompassed random sequencing.[2]

Stanford agreed that the claims were directed to targeted sequencing. JA003802. Stanford’s litigation counsel even agreed that “the two patents[3] [discussed at the claim construction hearing] are both targeted sequencing.” JA004186. The sole claim construction dispute was whether the claims of the ’076

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[2] It was not until the IPR that Ariosa first argued that the claims encompass the opposite, random approach. *See, e.g.*, JA000109 (“An alternative interpretation of “sequencing predefined subsequences” would be ‘shotgun sequencing of random fragments from a sample which include sample molecules those having predefined sequences’”); JA001214-15 (Ariosa’s expert stating, “the most reasonable interpretation based on my reading of the patent is that the claims are directed to shotgun sequencing methods…”).

[3] The two patents discussed at the claim construction hearing were the ’076 patent at issue in this appeal, and the ’430 patent, which is the subject of two separate appeals before this Court, i.e., 15-1215 and 15-1226.

patent are limited to a single method of targeted sequencing – a selective capture method that excludes targeted massively parallel sequencing. JA004226. Ariosa argued for the narrow interpretation that the claims only encompassed a single method, whereas Stanford argued the claims encompassed all of the targeted sequencing embodiments disclosed in the '076 patent. JA002161-63. The district court agreed with Stanford that although the claims are limited to targeted sequencing, they are not limited to a single method of targeted sequencing. *Id.*

III. Summary of the Proceedings in IPR2013-00308

This appeal is taken from the Final Written Decision in *Inter Partes* Review Case No. IPR2013-00308. The following is a brief summary of the proceedings before the PTAB.

A. Decision to Institute

Ariosa filed a Petition for *Inter Partes* Review of the '076 Patent ("the Petition") on May 24, 2013.[4] The Petition was supported by Declarations from two witnesses: Dr. Cynthia Morton and Dr. Robert Nussbaum. Based on the Petition and the witness declarations, the PTAB instituted *Inter Partes* Review of the '076 patent in a decision dated November 20, 2013 ("the Institution Decision").

[4] The Petition included multiple grounds of invalidity that were not instituted by the PTAB. JA000197. One additional ground was instituted, but the PTAB ultimately determined Ariosa failed to demonstrate that the '076 patent claims were unpatentable under this additional ground. JA000035. Those grounds that were not instituted, or were found unpersuasive, will not be discussed further.

In the Petition, Ariosa proposed two potential constructions for the term "sequencing predefined subsequences," that each utilized a different sequencing instrumentation and associated methodology. JA000107-109. One of the proposed constructions was specifically directed to random massively parallel sequencing, and is directly contrary to the construction Ariosa advocated in the district court. JA000109. The other proposed construction limited the term to the same selective capture method that the district court rejected as too narrow. JA000108; JA002161-63.

In the Institution Decision, the PTAB declined to adopt either of Ariosa's proposed constructions. JA000183. Instead**,** despite the claim term reading "sequencing ***predefined*** subsequences," and the contrast between targeted sequencing and random sequencing methods in the specification (JA000074 at 14:21-27), the PTAB determined that the claims encompassed any type of sequencing technique, including random sequencing. JA000182-83. Thus, the PTAB interpreted "sequencing predefined subsequences" to mean "sequencing predefined nucleic acid molecules that uniquely map to a chromosome region of interest in a reference genome." JA000183.

Based largely on its broad construction of "sequencing predefined subsequences" the PTAB found a reasonable likelihood that Ariosa would prevail

in establishing that claims 1 – 5, 7 – 9, and 12 – 13 are anticipated by Lo.[5] JA000185-86. The PTAB also found a reasonable likelihood that claim 6 was obvious in view of the combination of Lo and Li,[6] and that claims 10 and 11 were obvious in view of the combination of Lo and Brenner.[7] Accordingly, the PTAB instituted review of claims 1 – 13 of the '076 patent.[8]

B. The Lo Reference

The Lo reference is entitled "Diagnosing Fetal Chromosomal Aneuploidy Using Massively Parallel Genomic Sequencing." The sequencing method disclosed in the Lo reference is random massively parallel sequencing. For example, Lo states that "massively parallel sequencing is not dependent on the detection or analysis of predetermined or a predefined set of DNA sequences," instead "[a] random representative fraction of DNA molecules from the specimen pool is sequenced." JA001018. As an example, Lo states that the random fraction sequenced can represent 0.1%, 0.5%, 1%, 5%, 10%, 20%, or 30% of the genome.

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5 Lo et al., U.S. Patent Publication No. 2009/0029377, published January 29, 2009. ("Lo").

6 Heng Li et al., *Mapping Short DNA Sequencing Reads and Calling Variants Using Mapping Quality Scores,* 18 GENOME RESEARCH 1851 – 1856 (2008) ("Li").

7 Brenner, U.S. Patent Publication No. 2006/0177832, published August 10, 2006 ("Brenner").

8 Claims 14 and 15 of the '076 patent were not challenged.

JA001016. Lo further defines "random sequencing" as "sequencing whereby the nucleic acid fragments sequenced have not been specifically identified or targeted before the sequencing procedure." JA001013. For this reason, "[s]equence-specific primers to target specific gene loci are not required." *Id.*

Lo teaches that it is cost-effective to enrich a pool of nucleic acids to be sequenced prior to subjecting a fraction of that pool to random sequencing. JA001015-16. Thus, Lo discloses methods of enriching nucleic acid pools, such as with the use of an oligonucleotide hybridization array to "sub-select for nucleic acid sequences from certain chromosomes." JA001015. But Lo only describes subjecting the enriched pool to random sequencing. JA001015-16.

Lo does not disclose a method of targeted sequencing, i.e., sequencing only specific, predefined sequences within a chromosome, and actually contrasts the random sequencing method it does disclose with targeted sequencing methods. JA001011; JA001018. For example, Lo states that targeted sequencing methods may be less accurate "due to the small number of data points and typical statistical analysis." JA001011.

C. The PTAB's Final Written Decision

Relying principally on its earlier mistaken construction of "sequencing predefined subsequences," the PTAB's Decision, dated November 19, 2014, determined that claims 1-5, 7-9, 12-13 of the '076 patent were anticipated under 35

U.S.C. §102(e) by the Lo reference. JA000027. The PTAB also concluded that claim 6 was obvious over the combination of Lo and Li (JA000035), and that claims 10 and 11 were obvious over the combination of Lo and Brenner.[9]

Despite all of the contrary evidence, the PTAB maintained its unreasonable interpretation of "sequencing predefined subsequences" set forth in the Institution Decision. JA000013. The PTAB specifically found that the term is not limited to targeted sequencing methods based primarily on the specification's disclosure of random sequencing and the doctrine of claim differentiation. JA000010-12. The PTAB also relied upon the specification's disclosure of the concept of "predefined windows" to support its interpretation, even though neither party introduced evidence on this point. *Id.*

Despite the clear disclosure of targeted sequencing embodiments in the '076 specification (JA000074), the PTAB's Decision placed undue emphasis on the fact that the literal phrase "targeted sequencing" does not appear in the '076 specification. JA000010. The PTAB's Decision failed to address the specific disclosure in the '076 patent of methods that involve "sequencing selected

[9] The PTAB's Decision noted that Stanford offered no independent evidence or argument that those claims were patentable over the cited combination. JA000027-28; JA000035. Stanford instead relied on the evidence demonstrating that Lo does not anticipate claim 1, and thus all of the elements of claims 6, 10, and 11 are not taught by the cited combination. *See* 35 U.S.C. § 112, ¶ 4 (pre-AIA).

subsequences," or that those sequencing embodiments are "contrary to conventional massively parallel sequencing." JA000074 at 14:21-22.

Applying its unreasonable construction, the PTAB found that Lo's disclosure of random sequencing anticipates the '076 patent claims. JA000020. The PTAB also found that Lo teaches the use of hybridization arrays to enrich pools of nucleic acid sequences from certain chromosomes prior to subjecting them to random sequencing. JA000021. The PTAB found that Lo's disclosure of enriching pools for subsequent random sequencing constitutes a pre-selection step, even though it also found that the '076 claims do not require pre-selection. *Id.*

The PTAB's Decision failed to address numerous pieces of evidence submitted by Stanford to establish the claimed method is limited to targeted sequencing, and, as a result, is not anticipated by Lo. For example, the PTAB's Decision does not address:

- The afore-mentioned disclosures of the '076 specification of targeted sequencing embodiments (JA000074 at 13:53-14:27), and the contrast between those embodiments and random sequencing (JA000074 at 14:21-25);
- Admissions made by Dr. Morton in her first deposition that the claims of the '076 patent do not encompass random sequencing embodiments (JA001918-19; JA001941);
- Dr. Detter's testimony that the claims do not encompass random sequencing (JA3657-61);

- Ariosa's admissions in the district court that the claims are limited to targeted sequencing (*See*, Statement of the Case, Section, II, *supra*);

- Dr. Morton's testimony that Lo does not teach targeted sequencing (JA001967-68);

- Dr. Detter's confirmatory testimony that Lo does not teach targeted sequencing (JA003666-673).

Finally, the PTAB did not address a Stanford's Motion to Exclude the second Morton declaration (JA000413-31), except as it pertained to Stanford's objection to paragraph 15, which contained an erroneous figure and an expanded discussion of Lo paragraph 72. JA000036. However, despite Dr. Morton's admission that paragraph 15 contains a mistake (JA003271-74), the PTAB not only declined to exclude it, but indeed relied upon it. JA000036-37.

SUMMARY OF THE ARGUMENT

The PTAB's application of an overbroad and unreasonable construction of the claim term "sequencing predefined subsequences" caused the PTAB to make the wrong decision. The PTAB's construction of this term is contrary to the plain meaning of the claim language, the '076 patent specification, and the expert testimony of both parties. In reaching its erroneous construction, the PTAB ignored the expert evidence submitted by Stanford's expert, Dr. Detter, and admissions of Dr. Morton that confirm the '076 claims do not encompass random sequencing. Instead, the PTAB looked to a separate disclosure in the '076 patent

that was not the subject of any expert testimony, and does not control the proper interpretation of the '076 claims. As such, this Court should set aside the PTAB's erroneous claim construction, and instead find the '076 claims are limited to targeted sequencing.

The word "predefined" in the phrase "sequencing predefined subsequences" makes it clear that the '076 patent claims are directed to targeted sequencing. The remainder of step (b) is also clear that the purpose of sequencing the predefined subsequences is to obtain sequence tags that align only to those predefined subsequences. Thus, the selection step must occur ***before*** the sequencing step. Yet the PTAB asserts that the "pre-selection" can instead occur through an informational selection step that occurs ***after*** the sequencing step. To reach this determination, the PTAB reads in an unrelated disclosure of "predefined windows" from the specification. But the disclosure of "predefined windows" has no bearing on the targeted sequencing methods claimed in the '076 patent. Indeed, windows are not even mentioned in the section of the specification that describes targeted sequencing.

The specification of the '076 patent makes it clear that targeted sequencing methods are contrary to random sequencing methods. In targeted sequencing, a pre-selection step is employed to ensure that only specific, predefined sequences are molecularly sequencing. This is in direct contrast to random sequencing,

which sequences DNA fragments in a sample indiscriminately. Yet the PTAB improperly reads these random sequencing embodiments into the '076 claims. In doing so, the PTAB ignored all of the contrary evidence, including the testimony of Ariosa's own expert. That the specific phrase "targeted sequencing" does not literally appear in the '076 specification does not mean that the concept of targeted sequencing is not disclosed. Indeed, the disclosure of targeted sequencing was recognized by those of skill in the art, and by Ariosa itself in the district court.

The record before the PTAB includes the claim language itself, the specific disclosure of targeted sequencing in the specification, the testimony of both parties' experts interpreting the intrinsic evidence, and the admissions of Ariosa in the district court. All of this evidence leads to a clear conclusion that the '076 claims encompass only targeted sequencing. It was thus clear error for the PTAB to disregard the record and unreasonably construe the claims as encompassing random sequencing.

When the proper construction of the term is given, limiting it to targeted sequencing, the PTAB's invalidity analysis falls apart because Lo only discloses random sequencing. In fact, Ariosa's own expert admitted Lo does not teach targeted sequencing. Moreover, Lo's disclosure of enriching a pool of nucleic acids for the purpose of conducting ***random*** sequencing is not a substitute for the

pre-selection step of targeted sequencing. Thus, Lo cannot anticipate the '076 claims, and it was error for the PTAB to conclude otherwise.

ARGUMENT

I. Standard of Review

A. Claim Construction

The underlying factual determinations of the PTAB's claim construction that concern extrinsic evidence are reviewed for substantial evidence, and the ultimate construction of the claim is reviewed de novo. *In re Cuozzo Speed Tech., LLC,* 778 F.3d 1271, 1282-83 (Fed. Cir. 2015) (citing *Teva Pharms. U.S.A., Inc. v. Sandoz, Inc.,* 135 S. Ct. 831, 841 (2015)). If there is no issue as to extrinsic evidence, this Court reviews claim construction de novo. *Id.*

B. Validity

This Court reviews the PTAB's factual findings for substantial evidence, and reviews its legal conclusions de novo. *In re Baxter Int'l, Inc.*, 678 F.3d 1357, 1361 (Fed. Cir. 2012). Anticipation is a question of fact reviewed for substantial evidence. *In re Morsa,* 713 F.3d 104, 109 (Fed. Cir. 2013).

II. The PTAB Erred in Construing the Term "Sequencing Predefined Subsequences"

In an IPR, "a claim in an unexpired patent shall be given its broadest reasonable construction in light of the specification of the patent in which it appears." 37 C.F.R. § 42.100(b). However, the broadest reasonable construction

rubric does not give an unfettered license to interpret claims to embrace anything remotely related to the claimed invention. *In re Suitco Surface,* 603 F.3d 1255, 1260 (Fed. Cir. 2010). Claims should be read in light of the specification and teachings in the underlying patent. *Id.* (citing *Schriber-Schroth Co. v. Cleveland Trust Co.*, 311 U.S. 211, 217 (1940)). Indeed, the claims themselves provide substantial guidance as to the meaning of particular claim terms. *Philips v. AWH Corp.,* 415 F.3d 1303, 1314 (Fed. Cir. 2005). However, "the claims of a patent need not encompass all disclosed embodiments." *TIP Sys., LLC v. Phillips & Brooks/Gladwin, Inc.,* 529 F.3d 1364, 1373 (Fed. Cir. 2008). Moreover, "all claim terms are presumed to have meaning in a claim." *InnovaPure Water, Inc. v. Safari Water Filtration Sys., Inc.,* 381 F.3d 1111, 1119 (Fed. Cir. 2004).

The PTAB failed to apply this law in its analysis. As a result, the PTAB unreasonably construed the claims of the '076 patent to encompass all sequencing embodiments disclosed in the '076 patent specification. In particular, the PTAB erroneously determined that the claims encompass random sequencing. JA000013. In reaching this determination, the PTAB made at least the following errors:

- ignored the clear language of the claims;
- ignored the disclosure of targeted sequencing embodiments in the specification;
- adopted a claim interpretation that encompasses both targeted and random sequencing embodiments despite the specification's clear

statement that targeted sequencing is "contrary to" random sequencing;

- supported its construction with reference to a separate disclosure of "predefined windows" that was not advocated or supported by evidence introduced by either party;
- ignored the testimony of both Stanford's and Ariosa's expert witnesses that was contrary to its adopted claim construction;
- ignored Ariosa's admissions to the district court that the '076 claims do not encompass random sequencing; and,
- improperly applied the doctrine of claim differentiation.

The PTAB committed legal error in construing the claims. Stanford thus requests that this Court reject the PTAB's overly broad and unreasonable construction of the claim term "sequencing predefined subsequences," and reverse the PTAB's invalidity determination. Because the PTAB did not rely on extrinsic evidence to reach its erroneous conclusion, this Court may review the PTAB's claim interpretation *de novo.*

A. The Claims of the '076 Patent Are Directed to Targeted Sequencing

The '076 patent describes two approaches to sequencing: random and targeted. Importantly, the specification explicitly states that the targeted approach is *contrary* to the random approach. JA000074 at 14:21-27. In the targeted approach specific subsequences are ***predefined*** (pre-selected) prior to sequencing. JA003650-51. This is the key distinction between random and targeted

sequencing, and a person of ordinary skill in the art would understand these techniques are not equivalent. *Id.* This is also the approach reflected in the claims, which require the sequencing of only "predefined subsequences."

1. Inclusion of the Term "Predefined" in Step (b) Confirms that the '076 Patent Claims Are Limited to Targeted Sequencing

The challenged claims of the '076 patent include the claim term "sequencing ***predefined*** subsequences." The plain language of this term is clear - only "predefined" subsequences are sequenced. Ariosa's own attorneys admitted this in the district court:

> Now, let's begin with the claim itself. The claim says you're sequencing - - again, very important as far as the sequencing step. ***You're sequencing what? Predefined subsequences. Again, this is a targeted approach.*** They're subsequences. Second, they're not just any subsequences. They're predefined. Selected a priori.

JA004222-23 (emphasis added). But the PTAB's interpretation fails to give the term this plain meaning.

The PTAB interprets this term as encompassing any sequencing technique, including random sequencing. By construing the claim term to encompass any sequencing, the PTAB effectively reads the word "predefined" out of the claims, i.e., under the PTAB's construction the claim is read as though it states "sequencing sequences." This is error because all claim terms are presumed to

have meaning. *InnovaPure Water,* 381 F.3d at 1119. Moreover, rather than encompassing random sequencing, the claims actually exclude it. Indeed, the plain and ordinary meaning of the term "predefined," confirms to a person of ordinary skill in the art that the subsequences recited in the claim are already known, and targeted, prior to generating the sequencing output. JA003676.

The distinction between random sequencing and targeted sequencing is stark. In random shotgun sequencing, the nucleic acids in a sample are chosen randomly and sequenced. JA003650. There is no pre-selection step. *Id.* In contrast, targeted sequencing utilizes a pre-selection step so that only specific ***predefined*** sequences are actually sequenced. *Id.* In other words, any sequence that has not been pre-selected for sequencing is simply not sequenced. This understanding is consistent with the plain meaning of the term, and is supported by the testimony of expert witnesses of both parties.

i. The Remainder of Step (b) Confirms the Sequencing is to Obtain ONLY Those Sequence Tags that Align to the Predefined Subsequences

Interpreting the term "sequencing predefined subsequences" to exclude random sequencing, which is indiscriminate sequencing, is also consistent with the remainder of step (b). Step (b) describes that the purpose of sequencing the predefined subsequences is:

> 1. (a) …
> (b) sequencing predefined subsequences of the maternal and fetal DNA ***to obtain*** a plurality of ***sequence tags aligning to the predefined subsequences***, wherein said sequence tags are of sufficient length to be assigned to a specific predefined subsequence, wherein the predefined subsequences are from a plurality of different chromosomes, and wherein said plurality of different chromosomes comprise at least one first chromosome suspected of having an abnormal distribution in said sample and at least one second chromosome presumed to be normally distributed in said sample;
> (c) …

As is clear from the emphasized portions of step (b), the purpose of sequencing predefined subsequences is to obtain sequence tags that align to ***the*** predefined subsequences that were sequenced. If the sequencing step was indiscriminate - as it is in random sequencing - sequence tags that fail to align to the predefined subsequences would also be obtained. But that is at odds with step (b), which requires the obtained sequence tags to be aligned with, and assigned to, the predefined subsequences that were sequenced. JA003659-60. Interpreting "sequencing predefined subsequences" to exclude random sequencing is in accordance with its use in step (b), and is consistent with how a person of ordinary skill in the art understands the term. *Id.*

The PTAB fails to address this claim language in anything other than a superficial fashion. For instance, the PTAB notes that sequencing is associated with obtaining sequence tags, but fails to acknowledge that in the '076 claims the

only sequence tags obtained are those that align to the predefined subsequences. JA000010. Instead, the PTAB associates the formation of sequence tags exclusively with random sequencing, which generates sequence tags that align to sequences other than the predefined subsequences. *Id.* ("The Specification of the '076 patent discusses sequence tags in the context of shotgun sequencing.") (internal citations omitted). But the PTAB's restrictive association is flawed. Use of massively parallel sequencing for targeted sequencing will result in the formation of sequence tags, just as it does in random sequencing.[10] Accordingly, the PTAB's association of sequence tags with only random sequencing in its construction of "sequencing predefined subsequences" constitutes clear error, because it ignores the remaining claim language of step (b).

ii. The PTAB's Association of "Predefined Windows" with Targeted Sequencing is Baseless

The PTAB attempts to reconcile its unreasonable interpretation of "sequencing predefined subsequences" by stating that the sequences can be predefined in an informational sense, rather than in a molecular sense. JA000013. As support the PTAB's Decision references the use of "predefined windows." But this disclosure concerns a separate invention that is the subject of a separate patent

[10] The experts for both parties agree that massively parallel sequencing platforms can be used for targeted sequencing. JA003651; JA001366-67; JA001191.

(U.S. Patent No. 8,195,415)[11], and is irrelevant to the construction of "sequencing predefined subsequences." *See Ventana Med. Sys., Inc. v. Biogenex Labs, Inc.,* 473 F.3d 1173, 1181 (Fed. Cir. 2006) ("each claim does not necessarily cover every feature disclosed in the specification. When the claim addresses only some of the features disclosed in the specification, it is improper to limit the claim to other unclaimed features."). The PTAB erred by importing this unrelated embodiment into claim 1 of the '076 patent. Claim 1 does not suggest – much less claim – the use of windows. Moreover, in describing the targeted sequencing embodiments, the '076 patent specification does not so much as mention the use of windows. *See, e.g.*, JA000074.

Compounding this error, the PTAB cited no evidence to support its position that step (b) should be interpreted as an informational pre-selection step. Indeed, no evidence was ever introduced to suggest the use of predefined windows as a means for "sequencing predefined subsequences." This is not surprising because the use of "predefined windows" to analyze sequence tags generated by shotgun sequencing relates to a separate invention that is unrelated to the claims of the '076 patent.

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[11] The PTAB has reviewed the claims of the '415 patent in IPR2013-00390. In that proceeding, the PTAB described the '415 patent as describing the use of shotgun sequencing followed by mapping of the DNA to detect fetal aneuploidy. *Sequenom, Inc. v. The Board of Trustees of the Leland Stanford Jr. Univ.,* IPR2013-00390, Paper No. 45 at 4-5 (PTAB Nov. 25, 2014).

Step (b) is properly restricted to a molecular pre-selection step, and does not encompass an informational pre-selection of sequences. As described above, to find otherwise ignores that the predefined subsequences are sequenced to obtain specific sequence tags that align only to predefined subsequences. Concluding that step (b) is an informational selection step simply ignores the language of the claim, and is error.

2. The Plain Language of Steps (c) and (d) Confirms Stanford's Interpretation of "Sequencing Predefined Subsequences"

Subsequent steps of claim 1 further confirm that the claims are limited to sequencing ***only*** predefined subsequences. For example, steps (c) and (d) of claim 1 indicate that the claims are limited to targeted sequencing:

> 1. (a) …
> (b) …
> (c) ***assigning*** the plurality of ***sequence tags to*** their ***corresponding predetermined subsequences***;
> (d) ***determining a number of sequence tags aligning to the predetermined subsequences*** of said first chromosome and a number of sequence tags to the predetermined subsequences of the second chromosome…
> (e) …

Each of these steps requires assigning the sequence tags to the predetermined subsequences. That the term "subsequences" is used in these steps indicates that only part of an entire sequence (i.e., certainly less than an entire chromosome) is being sequenced. Moreover, the fact that the sequence tags are

assigned to "predetermined" locations further confirms that only particular subsequences have been sequenced, which is the very concept of targeted sequencing.

The PTAB essentially ignores these claim steps. Rather, the PTAB merely repeats the claim language of steps (c) and (d), and states "[s]teps (c) and (d) of claim 1 are not inconsistent with our construction." JA000012. The PTAB's reasoning on this point appears to be largely derived from its incorrect interpretation that the '076 patent claims are not restricted to the molecular sequencing of predefined subsequences. But this is clear error because it is contrary to the language of the claims, and the disclosure of the specification. (See Argument, Section II(B), *infra*).

B. The Specification Distinguishes Targeted Sequencing from Random Sequencing

The '076 patent specification describes targeted sequencing approaches. *See, e.g.*, JA000074 at 13:54-56 ("One may use sequencing methods which select a priori sequences which map to the chromosomes of interest."); *Id.* at 13:65 ("In sequencing selected subsequences…"). Importantly, the '076 specification specifically differentiates targeted sequencing from random (conventional) massively parallel sequencing:

> The [targeted] ***subsequencing method is in one aspect contrary to conventional massively parallel sequencing*** methodologies, which seek to obtain all of the sequence information in a sample. ***This***

> ***alternative method selectively*** ignores certain sequence information by using a ***sequencing*** method which selectively captures sample molecules containing certain ***pre-defined sequences***.

Id. at 14:21-27 (emphasis added). A person of ordinary skill in the art would have known from these disclosures that the '076 patent is describing targeted sequencing, and specifically contrasting it with random sequencing. JA003660-61. Because the claim term states "sequencing ***predefined subsequences***," a person of ordinary skill would have understood that it is these targeted sequencing embodiments that are being claimed in the '076 patent.

The '076 specification is explicit that targeted sequencing is distinct from, and in contrast to, random sequencing. JA000074 at 14:21-27. But the PTAB failed to recognize the distinction, and this led it to rely on random sequencing embodiments in construing the claims. For example, the PTAB refers to the examples in the '076 patent that refer to shotgun, i.e., random, sequencing. JA000011. Although the '076 patent includes examples of random sequencing, it also discusses embodiments that are contrary to random sequencing. JA000074 at 14:21-27. These are the embodiments claimed in the '076 patent, and it was error for the PTAB to read the random sequencing embodiments into the claims of the '076 patent.

1. The Concept of Targeted Sequencing is Explicitly Disclosed as a Preferred Embodiment

The PTAB's Decision also incorrectly asserts that the preferred embodiment of the '076 patent involves shotgun sequencing. JA000011-12. This is not so. Targeted sequencing is clearly described as a preferred embodiment, and it was error for the PTAB to suggest that the preferred embodiment must involve random sequencing.

The "Detailed Description of the Preferred Embodiment" section in the '076 patent discusses sequencing embodiments that are "contrary to conventional massively parallel sequencing." JA000074 at 14:21-27. In further describing these embodiments, the '076 patent notes that one embodiment involves using a "sequencing method which selectively captures sample molecules containing certain predefined sequences." *Id.* These sequencing methods, "select a priori sequences which map to the chromosome of interest…" JA000074 at 13:54-57. The specification even discloses specific methods other than massively parallel sequencing that are suitable for "sequencing selected subsequences," e.g., sequencing by array, and capture beads. JA000074 at 13:65-14:1. A person of ordinary skill in the art would have known from these disclosures that these preferred embodiments are describing the concept of targeted sequencing. JA003660-61.

The PTAB did not address these disclosures, nor the evidence introduced regarding the understanding of a person of ordinary skill. Rather, the PTAB's analysis of the targeted sequencing disclosures in the specification starts and stops with its conclusion that the term "targeted sequencing" does not literally appear in the specification. JA000010. That may be, but it does not change the specification's explicit disclosure of the concept of targeted sequencing. It certainly does not change the understanding of one of ordinary skill in reading the specification. Nor does it change the testimony of Ariosa's expert in the IPR, or Ariosa's statements to the district court, that the claims are directed to targeted sequencing. The PTAB's failure to consider this evidence constitutes error.

2. The Claim Terms are Used in the Specification to Describe Targeted Sequencing

The specification uses the terms "predefined sequences" and "subsequences" only in describing targeted sequencing embodiments. Even the PTAB recognized that the '076 specification discloses sequencing methods "that may be used to sequence ***selected subsequences***…" JA000004. The PTAB also recognized that the specification teaches a "***subsequencing*** method" that "selectively ignores certain sequencing information by using a sequencing method which selectively captures sample molecules containing certain ***predefined sequences***…" JA000005 (emphasis added). Not surprisingly, in discussing the supposedly "preferred" random sequencing embodiment, the PTAB failed to cite to similar terms.

JA000010-13. And for good reason, such terms are not used in conjunction with random sequencing. Thus, the PTAB's construction is inconsistent with the specification, and is contrary to the PTAB's requirement to construe claim terms "in light of the specification of the patent in which they appear." 37 C.F.R. § 42.100(b); *See also In re Suitco Surface,* 603 F.3d at 1260.

C. The PTAB's Claim Construction is Unsupported by the Evidentiary Record Before It

When the AIA replaced inter partes reexamination with inter partes review it was expressly noted that the intent was to convert the process from an examinational proceeding to an adjudicative proceeding. H.R. Rep. No. 112-98, pt. 1, 46-47 (June 11, 2011) ("The Act converts inter partes reexamination from an examination to an adjudicative proceeding, and renames the proceeding 'inter partes review.'"). This conversion has been recognized by this Court. *Abbott Labs v. Cordis, Corp.,* 710 F.3d 1318, 1326 (Fed. Cir. 2013). The PTAB itself has recognized that '[a]n inter partes review is neither a patent examination nor a patent reexamination," but is "a trial, adjudicatory in nature and constitutes litigation." *Google Inc. v. Jongerius Panoramic Techs., LLC,* IPR2013-00191, Paper No. 50, at 4 (PTAB Feb. 13, 2014) (granting *pro hac vice* motion for litigation counsel to participate in the IPR). As such, the PTAB must adhere to the evidentiary standards required of a district court. *See In re Lee,* 277 F.3d 1338, 1344 (Fed. Cir. 2002) ("Deferential judicial review under the Administrative

Procedure Act does not relieve the agency of its obligation to develop an evidentiary basis for its findings.")

Three experts – Drs. Morton, Nussbaum for Ariosa, and Detter for Stanford – provided evidence in this case that reflects their understanding of the term "sequencing predefined subsequences." The testimony of all three of these experts is contrary to the interpretation the PTAB ultimately reached. Yet the PTAB disregarded all of this contrary evidence in construing the claims.[12] This constitutes error; and, because the PTAB's error includes this disregard of the extrinsic evidence, this Court may review the PTAB's claim construction *de novo.*

Dr. Morton, one of Ariosa's experts, testified that it was her opinion that the claimed method is a "directed sequencing method," and that "directed sequencing is not random." JA001917; JA001922. Dr. Morton further testified it was her understanding that the '076 patent claims do not claim "sequencing all sequences in terms of sequencing the entire human genome, which I think [refers to] shotgun massively parallel sequencing." JA001918-19. Rather it was her understanding that in the '076 patent claims, "you choose the sequences that you would like to use to base your further conclusions on before you do the sequencing. So you're sequencing a selected group of sequences." *Id.*

[12] As discussed in Argument, Section II(A)-(B), *supra,* the PTAB also ignored the language of the claims, and the disclosures in the specification.

Dr. Morton's testimony is clear. The '076 patent claims are directed to a method of targeted sequencing, and do not encompass random sequencing.[13] Dr. Morton's testimony on this point is particularly noteworthy given that it is in direct conflict with her first declaration in the IPR, which asserted "the most reasonable interpretation based on my reading of the patent is that the claims are directed to shotgun sequencing methods…." JA001214-15. That she could not defend this position on cross-examination is telling, and should not have been ignored by the PTAB.

Dr. Nussbaum, Ariosa's other expert, agrees that step (b) involves a physical pre-selection. JA001375 ("predefined subsequences…refers to a physical molecule subject to a sequencing operation…"); JA001375-76 ("the element 'predefined subsequence' in the claims, however, is itself subjected to sequencing and thus must be a nucleic acid rather than a bioinformatic parameter."). Dr. Nussbaum's testimony is inconsistent with the PTAB's finding that the claims include "informationally predefining the subsequences." JA000013. Stanford never disagreed with Dr. Nussbaum on this point. Because this evidence went unrebutted, the PTAB's opposite conclusion is clearly not supported by any evidence.

[13] Notably, Dr. Morton's characterization of the claims is the same as Ariosa's characterization in the district court.

Dr. Detter - Stanford's only expert – also testified that a person of ordinary skill in the art would have understood the claim term "sequencing predefined subsequences" to be restricted to targeted sequencing. JA003658-61. This testimony was based on a reasoned analysis of the claim language, and the specification. *Id.* Importantly, Dr. Detter's analysis gives the term "predefined" its proper weight. As explained by Dr. Detter, based on the context of its use in the '076 patent claims, a person of ordinary skill in the art would understand that the term "predefined" refers to preselecting the nucleic acids to be sequenced prior to sequencing them. JA003659. This excludes random sequencing. JA003661.

The PTAB erred by ignoring this testimony. Instead, the PTAB elected to adopt a definition of "sequencing predefined subsequences" that includes random molecular sequencing with informationally predefined sequences. JA000013. But the PTAB cited no evidence for this interpretation. *See In re Zurko,* 258 F.3d 1379, 386 (Fed. Cir. 2001) ("the Board cannot simply reach conclusions based on its own understanding…. Rather, the Board must point to some concrete evidence in the record in support."). Indeed, the PTAB's interpretation is contrary to the testimony of the experts of both parties, and is controverted by the clear language of the claims. As such, the PTAB's construction in this case is not supported by an evidentiary basis, and is at odds with the adjudicative nature of an IPR.

D. The PTAB Erred by Failing to Take into Ariosa's Admissions in the District Court into Account

In the related patent infringement litigation, Ariosa admitted the claims of the '076 patent were limited to targeted sequencing. *See,* Statement of the Case, Section II, *supra.* This position is directly contrary to what Ariosa argued to the PTAB during the IPR. JA004222-23. Moreover, Ariosa's change in position has no tangible connection to any differences between claim construction standards before the PTAB versus the district court. How could it? Ariosa's new construction is the exact opposite of what it argued for in the district court. No rational argument can be made that the claims now encompass such diametrically opposed methods of sequencing simply because the claim construction standard has changed. *See In re Suitco Surface,* 603 F.3d at 1260 (" the broadest reasonable construction rubric coupled with the term 'comprising' does not give the PTO an unfettered license to interpret claims to embrace anything remotely related to the claimed invention").

That Ariosa directly stated in a different – but still adversarial – forum that the claims are limited to targeted sequencing is a powerful indicator that the claims are in fact limited to targeted sequencing. Notably, Stanford ***agreed*** with Ariosa that the claims should be limited to targeted sequencing. But the PTAB failed to take into account the parties' agreement that the claim were limited to targeted sequencing. This is yet another instance where the PTAB failed to act on the

evidence before it. Unlike the PTAB, this Court should take note of Ariosa's contrary admissions to the district court in reaching its own, independent interpretation of the claims. *See Cuozzo Speed Tech.,* 778 F.3d at 1282-83 (If there is no issue as to extrinsic evidence, this Court reviews claim construction de novo).

E. The PTAB Erred in Relying on Claim Differentiation

The PTAB relied in part on the doctrine of claim differentiation to reach its overbroad interpretation of the term "sequencing predefined subsequences." JA000012-13. However, the doctrine of claim differentiation is merely a presumption, and it cannot overrule the clear disclosure of the specification. *Tate Access Floors, Inc. v. Maxcess Tech., Inc.* 222 F.3d 958, 968 (Fed. Cir. 2000) ("The doctrine of claim differentiation cannot broaden claims beyond their permissible scope"); *Multiform Desiccants, Inc. v. Medzam, Ltd.,* 133 F.3d 1472, 1480 (Fed. Cir. 1998) ("The doctrine of claim differentiation cannot broaden claims beyond their correct scope, determined in light of the specification…and any relevant extrinsic evidence."). In this case, the specification of the '076 patent clearly describes embodiments that involve only the selective sequencing of certain predefined subsequences. *See* Argument, Section II(B), *supra.* The experts also agree that the claims of the '076 patent are directed to targeted sequencing. *See* Argument, Section II(C), *supra.* In the face of this evidence, claim differentiation should be entitled to little or no weight.

In applying claim differentiation to support its interpretation of "sequencing predefined subsequences," the PTAB looked to claim 9 of the '076 patent.[14] Claim 9 specifies that the "sequencing comprises selectively sequencing nucleic acid molecules comprising the predefined sequences." The PTAB interpreted this limitation to mean that "the sequence step is limited to sequencing only the predefined subsequences," and thus the sequencing step of claim 1 must encompass sequences other than the predefined subsequences. However, even if the PTAB is correct, it was error for the PTAB to use this logic to broaden its interpretation of claim 1, but then ignore that same logic for purposes of validity as discussed in Argument, Section IV, *infra.*

III. Using the Proper Construction, the Lo Reference Does Not Anticipate

Using an overbroad and unreasonable interpretation of the term "sequencing predefined subsequences" that includes random sequencing embodiments, the PTAB found that the Lo reference anticipates the claims of the '076 patent. However, when that term is properly construed to exclude random sequencing embodiments, the Lo reference does not anticipate.

A. Lo Only Describes Random Sequencing

Lo does not teach how to preselect sequences for sequencing. Dr. Morton's testimony on this point is clear:

[14] Claim 9 depends from claim 2, which in turn depends from claim 1.

> Q: ***Lo does not teach how to preselect the molecules*** that are going to be sequenced does it?
> A: So I think that had to do in considering claims 10 and 11 in the Fan patent, and so - - because I - - ***I'm agreeing with you that that is not taught in Lo***…

JA001967-68 (emphasis added). Instead, Lo discloses only random sequencing, which does not include a pre-selection step. JA003670-71.

The PTAB relies on the Lo disclosure of massively parallel sequencing for the teaching of "sequencing predefined subsequences." JA000020. But the only use of massively parallel sequencing in Lo is directed to random sequencing:

> In one aspect for the massively parallel sequencing approach, representative data from all of the chromosomes may be generated at the same time. ***The origin of a particular fragment is not selected ahead of time. The sequencing is done at random*** and then a database search may be performed to see where a particular fragment is coming from. This is contrasted from situations when a specific fragment from chromosome 21 and another one from chromosome 1 are amplified.

JA001016 (emphasis added). This disclosure confirms that the sequencing methods of Lo do not utilize a pre-selection step such that only particular predefined subsequences are sequenced. In fact, the Lo reference defines random sequencing as "sequencing whereby the nucleic acid fragments sequenced ***have not been specifically identified or targeted*** before the sequencing procedure." JA001013 (emphasis added). Thus, the random sequencing described in Lo cannot satisfy a claim limitation that requires "sequencing ***predefined*** subsequences"

because it does not pre-select sequences of interest for sequencing. In other words, Lo does not disclose targeted sequencing.

Paragraph 80, which appears under the heading "Random Sequencing," introduces Figure 2 of the Lo publication as a flowchart for a method of performing prenatal diagnosis of a fetal chromosomal aneuploidy using random sequencing. JA001002; JA001016. In paragraph 82, Lo further explains that a feature of the random sequencing method referenced in paragraph 80,

> is that the nucleic acids to be sequenced ***are not specifically identified or targeted*** before the sample analysis, i.e., sequencing. Sequence-specific primers to target specific gene loci are not needed for sequencing.

JA001016 (emphasis added). A person of ordinary skill in the art would understand from this further description in the Lo publication that the random sequencing method of Lo does not sequence only predefined subsequences, because there is no prior identification or targeting of specific sequences. JA003672-73.

These representative disclosures of the Lo publication are contrary to the '076 claims because those claims explicitly require "sequencing ***predefined*** subsequences." JA003673-74. Thus, the '076 claims are dependent on sequencing only a "predefined" set of DNA sequences. By contrast, the Lo publication's definitions and disclosures specifically note that sequences are not selected ahead of time for sequencing, and the identities of the nucleic acids sequences are not

revealed until the sequencing output is generated. But the predefined subsequences recited in the '076 claims are already known, and specifically targeted, prior to generating sequencing output. JA003676; *See also,* Statement of the Case, Section I, *supra.* For at least these reasons, the random sequencing method described and defined by the Lo publication is not equivalent to sequencing predefined subsequences as required by the'076 patent claims. *Id.* Accordingly, the Lo publication does not anticipate the claims of the '076 patent.

B. The Lo Reference Does Not Describe Targeted Sequencing as Required by the '076 Claims

Although adamant that the '076 claims do not require a molecular pre-selection step, the PTAB asserts that to the extent claim 1 encompasses a pre-selection step, that step is also taught by Lo. JA000021. In reaching this conclusion, the PTAB relies on the disclosure of Lo paragraph 72:

> In another embodiment, the fraction of the nucleic acid pool that is sequenced in a run is further sub-selected prior to sequencing. For example, hybridization based techniques such as oligonucleotide array could be used to first sub-select for nucleic acid sequences from certain chromosomes, e.g., a potentially aneuploid chromosome and other chromosome(s) not involved in the aneuploidy tested.

JA000025; JA001015. But Lo paragraph 72 simply states that hybridization arrays can be used to select the fraction (i.e., all of the fragments) associated with a chromosome for sequencing. JA003668. This is ***not*** targeted sequencing. *Id.* The

PTAB's assertion to the contrary reflects a fundamental misunderstanding of the use of hybridization arrays, and the teachings of Lo.

Lo discloses the use of a hybridization array as a cost-saving measure (since every chromosome does not have to be counted in order to detect an aneuploidy) to pull down all of the fragments associated with a particular chromosome(s) prior to subjecting those fragments to random sequencing. JA001015-16; JA003668. For example, if only chromosomes 12 and 21 were to be used for the aneuploidy determination, Lo is teaching to limit the sequencing to chromosomes 12 and 21 by selecting out all of the chromosome 12 and 21 sequences. *Id.* Lo is not teaching to select just predefined subsequences within a chromosome of interest, as required by the '076 claims. *Id.* That is, Lo is not teaching to only select particular sequences of interest from particular chromosomes. This is confirmed by the example cited within the paragraph that specifically notes hybridization is used to sub-select potentially aneuploid chromosomes – not particular fragments within that chromosome. *Id.* However, using an array to select all fragments associated with a set of chromosomes is not the same concept as predefining subsequences for sequencing as required by the claims of the '076 patent.[15] *Id.*

[15] Ariosa may argue that Dr. Detter's testimony is unreliable by incorrectly asserting it is his belief that Lo refers to pulling down an intact chromosome. Ariosa's argument is without merit as evidenced by Dr. Detter's clear testimony that the Lo reference discusses pulling down all the fragments associated with a

Even Dr. Morton agreed that simply because a probe with a preselected sequence was used to pull down a fragment (like in a hybridization array) it does not mean that the part of the fragment that gets sequenced will be the part that matched the probe. JA003274-75. Dr. Morton also testified that use of massively parallel sequencing "will then generate sequences that weren't necessarily the sequences that were selected, because they happened to be attached to a longer fragment that was selected." JA003253-55. Accordingly, Dr. Morton acknowledges that simply using a hybridization array to pull down fragments, and subjecting those fragments to random sequencing (as described in Lo paragraphs 72 and 79) does not result in "sequencing predefined subsequences" of a chromosome as claimed in the '076 patent.

The hybridization array described in Lo paragraph 72 is further explained in Lo paragraph 79:[16]

> Alternatively, sequences originating from a potentially aneuploid chromosome and one or more chromosomes not involved in the aneuploidy could be enriched by hybridization techniques for example onto oligonucleotide microarrays. ***The enriched pools of nucleic acids would then be subjected to random sequencing.*** This would allow the reduction in sequencing costs.

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chromosome, and that intact chromosomes are not circulating in maternal blood. *See, e.g.*, JA003668; JA004270.

[16] Dr. Detter explained that Lo paragraphs 72 and 79 should be read together. JA003668-69. Dr. Morton agrees. JA003329-30.

JA001016 (emphasis added). A person of ordinary skill in the art would understand from the disclosure at Lo paragraph 79 that the purpose of isolating the chromosome is to subject it to "random sequencing," which would be inappropriate if the sequence had already been preselected. JA003669-70. Indeed, this would be inconsistent with Lo's definition of "random sequencing" wherein "the nucleic acid fragments have not been specifically identified or targeted before the sequencing procedure…" JA001013.

The '076 claims require that the predefined subsequence – and only the predefined subsequence – actually be sequenced. The use of a hybridization array as disclosed in Lo does not produce this result, and a person of ordinary skill in the art would have had this understanding. JA003668-69. Accordingly, the PTAB erred in concluding Lo paragraph 72 discloses targeted sequencing as claimed by the '076 patent.

C. Rather than Teach Targeted Sequencing, Lo Disparages it as Inaccurate

It is clear that Lo only discloses random sequencing, and does not disclose any method of targeted sequencing. JA003674. In fact, when discussing massively parallel sequencing, Lo states that the technique does ***not*** depend "on the detection or analysis of predetermined or a ***predefined*** set of DNA ***sequences***." JA001018 (emphasis added). Notably, the Background section of Lo disparages methods that only analyze sequences "selected prior to nucleic acid analysis" as

providing too few data points for accurate analysis in maternal samples. JA001016; JA003674. Given the criticism in Lo of using predefined subsequences (as claimed in the '076 patent), paragraph 72 of Lo should not be interpreted as teaching such a step. Moreover, the PTAB's assertion – based on reading paragraph 72 in isolation – that Lo does teach targeted sequencing, is inconsistent with the specification as a whole.

D. The PTAB Erred in Relying on the Second Morton Declaration

Ariosa submitted a second declaration from Dr. Morton to support its Reply.[17] Stanford moved to exclude this second declaration (JA000413-31) because it included new interpretations of the '076 patent claims and the Lo reference. Included with these new interpretations are instances where Dr. Morton's testimony completely changes from her first deposition to her second declaration.[18] Importantly, these changes occurred in her second declaration when

[17] The vast majority of the second Morton declaration was not cited by Ariosa in its Reply. Ariosa's counsel acknowledged that it contains additional explanation of various points that were not included in the Reply. JA000381. As such, the second Morton Declaration constitutes an unduly prejudicial way for Ariosa to avoid the page limits imposed by the rules. 37 C.F.R. § 42.24(c)(1).

[18] *Compare* First Morton deposition (JA001918 (stating that the claims of the '076 patent do not cover shotgun sequencing); JA001941 (stating that the '076 patent is not directed to random sequencing) *with* Second Morton Declaration (JA003068 (asserting the '076 patent does encompass random sequencing)).

neither Stanford nor its expert had the opportunity to meaningfully respond to these changed opinions.[19]

The second Morton declaration also contains a greatly expanded discussion of the Lo reference as compared to her first declaration.[20] This is exemplified by paragraph 15, which contains an extensive elaboration of Dr. Morton's opinion that Lo paragraph 72 teaches pre-selection. JA003061. The PTAB expressly relied on paragraph 15 in its validity analysis (JA000036), but paragraph 15 contains a mistake. JA003271-74 (Dr. Morton acknowledging that the Figure included in paragraph 15 is not accurate).

It was error for the PTAB to rely on paragraph 15 given that it contains a factual error. This is compounded by the PTAB's apparent disregard of Dr. Morton's prior admission that the Lo reference does not disclose a method for pre-selection (JA001967-68):

> Q: ***Lo does not teach how to preselect the molecules*** that are going to be sequenced does it?

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[19] For at least these reasons, it was an abuse of discretion to permit Ariosa to submit this Second Declaration over the repeated objections of Stanford, and then rely on this declaration in reaching its decision.

[20] *Compare* First Morton declaration, JA001217-19 (analysis of Lo paragraph 72 is reproduction of that paragraph and single conclusory sentence stating that it discloses a process where sequences are subselected prior to sequencing) *with* Second Morton declaration (JA003059-73 (Fourteen page analysis of Lo, particularly focused on Lo paragraph 72)).

> A: So I think that had to do in considering claims 10 and 11 in the Fan patent, and so - - because I - - ***I'm agreeing with you that that is not taught in Lo***…

JA001967-68 (emphasis added).

Dr. Morton's deposition testimony is clear - Lo does not teach a pre-selection step. *Id.* Dr. Detter agreed that Lo does not teach a pre-selection step, and he further explained why paragraph 72 does not teach the concept of targeted sequencing. JA003666-673. Ariosa attempted to rebut this evidence by submitting the second Morton declaration, but the PTAB did not rely on that second declaration, except for the admittedly erroneous paragraph 15. JA000036-37. As a result, the only evidence of record that Lo teaches a pre-selection step (in paragraph 15) is admittedly flawed. Accordingly, the prior testimony of Drs. Morton and Detter that Lo does not teach a pre-selection step is effectively unrebutted. Thus, there is no evidence – much less substantial evidence – in the record to support the PTAB's determination that Lo teaches a pre-selection step.

IV. The PTAB Committed Error By Failing to Separately Address the Validity of Claim 9

Claim 9 of the '076 patent further limits claim 1 by requiring that the "sequencing comprises selectively sequencing nucleic acid molecules comprising the predefined sequences." JA000085. As discussed above in Argument, Section II(D), the PTAB relied on claim 9 to support its construction via the doctrine of claim differentiation. In doing so, the PTAB specifically stated that in claim 9 "the

sequencing step is limited to sequencing only the predefined sequences." JA000012. It was error for the PTAB to use claim 9 to support its claim construction, but then fail to address claim 9 in its validity analysis.

Instead, the PTAB dodged the issue, and asserted that Patent Owner did not present separate argument as to claim 9. JA000026. Not true. During the oral hearing, Stanford explicitly called the PTAB's attention to claim 9 and noted that "[i]f for some reason you decided that Claim 1 wasn't limited as we described, Claim 9 certainly is limited in that same way." JA000613. The presiding judge responded "I understand that. Okay." *Id.* Thus there was no reason to further supplement the record when the judge clearly understood Stanford's argument that the language of claim 9 limited it to targeted sequencing.[21] In view of this record, it was error for the PTAB to assert that Stanford did not argue claim 9 separately. This error was compounded by the PTAB's refusal to consider the validity of claim 9 separately.

To the extent Ariosa will argue that Lo paragraph 72 anticipates claim 9, that argument must fail. The PTAB asserts that under its interpretation Lo paragraph 72 teaches "sequencing predefined subsequences" because "the oligonucleotides that make up the array are selecting for subsequences of the chromosome, which

[21] Ariosa's counsel also admitted at the final oral hearing that claim 9 is referring to targeted sequencing because of the "phraseology selectively sequencing nucleic acids comprising the predefined sequences." JA000618.

subsequences may then be analyzed using massively parallel sequencing." JA000025. But Dr. Morton testified that the use of hybridization arrays does not guarantee that the sequence that is pulled down is actually the part that is sequenced. JA003274-75.

Regardless, the PTAB acknowledges that Lo analyzes nucleic acid pools via massively parallel sequencing, but Lo only teaches ***random*** massively parallel sequencing. (*See* Argument, Section III(A), *supra*). However, Dr. Morton testified that use of massively parallel sequencing "will then generate sequences that weren't necessarily the sequences that were selected, because they happened to be attached to a longer fragment that was selected." JA003253-55. Thus, the Lo method disclosed in paragraph 72 ensures that the sequencing will not be limited to "selectively sequencing nucleic acid molecules comprising the predefined subsequences" as required by claim 9. Given these admissions it was error for the PTAB to hold claim 9 invalid with no explanation or factual findings to show how Lo meets the narrower limitation of claim 9.

V. The Properly Construed Claims of the '076 Patent are Not Obvious in View of the Cited Prior Art References

The PTAB concluded claims 10 and 11 of the '076 patent are obvious in view of the combination of Lo and Brenner. JA000027-28. The PTAB also concluded claim 6 of the '076 patent is obvious in view of the combination of Lo and Li. JA000035. The PTAB's Decision was based on Ariosa's contentions in

the Petition, as supported by the testimony of Dr. Morton. JA000027-28; JA000035. However, neither Ariosa nor the PTAB allege that the Brenner or Li references disclose anything other than the specific limitations recited by claims 6, 10 and 11. As such, the Lo reference is relied upon to teach the remaining elements. As discussed above, Lo does not anticipate these claims. Therefore, all of the elements of claims 6, 10 and 11 are not taught, and the claims are not obvious.

CONCLUSION

For the reasons discussed above, the PTAB's claim construction is erroneous and has no basis in the evidence of record. Because the PTAB did not rely on any extrinsic evidence to reach its conclusion, this Court may review that construction *de novo* and reach the correct interpretation. Under the correct interpretation, the claims of the '076 patent are not anticipated by the Lo reference. Accordingly, this Court should reverse the PTAB's findings of anticipation and obviousness.

Dated: May 4, 2015

Respectfully submitted,

/s/ R. Danny Huntington

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ADDENDUM 1

Final Written Decision, 35 U.S.C. § 318(a) and 37 C.F.R. § 42.73
November 19, 2014 (PTAB) (Paper 40)

JA000001 – JA000039

2015-1413
The Board of Trustees of the Leland Stanford Junior University
v.
Ariosa Diagnostics, Inc.

Trials@uspto.gov
571.272.7822

Paper No. 40
Filed: November 19, 2014

UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE PATENT TRIAL AND APPEAL BOARD

ARIOSA DIAGNOSTICS,
Petitioner,

v.

THE BOARD OF TRUSTEES OF THE LELAND STANFORD JUNIOR UNIVERSITY,
Patent Owner.

Case IPR2013-00308
Patent 8,296,076 B2

Before TONI R. SCHEINER, LORA M. GREEN, and SCOTT E. KAMHOLZ, *Administrative Patent Judges*.

GREEN, *Administrative Patent Judge*.

FINAL WRITTEN DECISION
35 U.S.C. § 318(a) and 37 C.F.R. § 42.73

I. INTRODUCTION

A. Background

Petitioner, Ariosa Diagnostics ("Ariosa"), filed a Petition requesting *inter partes* review of claims 1–13 ("the challenged claims") of U.S. Patent No. 8,296,076 B2 ("the '076 patent"). Paper 1 ("Pet."). Patent Owner, The

Board of Trustees of the Leland Stanford Junior University ("Stanford"), did not file a Patent Owner Preliminary Response. We determined that the information presented in the Petition demonstrated that there was a reasonable likelihood that Petitioner would prevail in challenging claims 1–13 as unpatentable under 35 U.S.C. § 102(e) and 35 U.S.C. § 103(a). Pursuant to 35 U.S.C. § 314, the Board instituted this proceeding on November 20, 2013, as to the challenged claims of the '076 patent. Paper 7 ("Institution Decision"; "Dec. Inst.").

Patent Owner filed a Response (Paper 15, "PO Resp."), but did not file a motion to amend. Petitioner subsequently filed a Reply. Paper 18 ("Reply"). An oral hearing was held on July 11, 2014. The transcript of the hearing has been entered into the record. Paper 39.

We have jurisdiction under 35 U.S.C. § 6(c). This final written decision is issued pursuant to 35 U.S.C. § 318(a). Based on the record before us, we conclude that Petitioner has demonstrated by a preponderance of the evidence that the challenged claims of the '076 patent are unpatentable.

B. Related Proceedings

The '076 patent is the subject of a civil action, *Verinata Health, Inc. v. Ariosa Diagnostics, Inc.*, No. 3:12-cv-05501-SI (N.D. Cal.), filed October 25, 2012. Paper 6.

C. The '076 Patent

The '076 patent issued on October 23, 2012, with Hei-Mun Christina Fan and Stephen R. Quake as the listed co-inventors. The '076 patent

"relates to the field of molecular diagnostics, and more particularly to the field of prenatal genetic diagnosis." Ex. 1001, col. 1, ll. 36–38.

The '076 patent teaches that fetal chromosomal aneuploidies may affect 9 out of every 1000 live births. *Id.* at col. 1, ll. 49–50. The discovery of significant amounts of cell-free nucleic acid in the maternal blood stream has led to the development of non-invasive prenatal tests for a variety of traits, but measurement of aneuploidy has presented a challenge due to the high background of maternal DNA, as fetal DNA often constitutes less than 10% of total DNA in maternal cell-free plasma. *Id.* at col. 1, l. 62–col. 2, l. 3. Methods that have been used to detect aneuploidy include detecting an allelic variation between the mother and the fetus, direct shotgun sequencing followed by mapping of fragments to the chromosome of origin, as well as enumeration of the number of fragments per chromosome. *Id.* at col. 2, ll. 4–21.

In a preferred method of the '076 patent, DNA is obtained from maternal serum, wherein the DNA is a mixture of maternal and fetal DNA. *Id.* at col. 3, ll. 40–47. The DNA is sequenced partially to provide a large number of short reads, which act as sequence tags, with a significant number of the short reads being sufficiently unique such that they can be mapped to specific chromosomes or chromosomal locations of the human genome. *Id.* at col. 3, 47–52. "By counting the number of sequence tags mapped to each chromosome (1–22, X and Y), the over- or under-representation of any chromosome or chromosome portion in the mixed DNA contributed by an aneuploid fetus can be detected." *Id.* at col. 3, ll. 54–58. As taught by the '076 patent, the method does not rely on *a priori* sequence information to distinguish fetal DNA from maternal DNA. *Id.* at col. 3, ll. 64–66.

The ’076 patent also discloses a method for correcting for the “nonuniform distribution [of] sequence tags to different chromosomal portions.” *Id.* at col. 4, ll. 54–55. The ’076 patent discloses a method in which a large number of windows of defined length are created along chromosomes of interest, such that the windows cover each chromosome of interest, except that non-informative regions of the chromosomes, such as centromere and repetitive regions, are not necessarily included. *Id.* at col. 4, ll. 56–62. As taught by the ’076 patent, “[v]arious average numbers, i.e., median values, are calculated for different windows and compared. By counting sequence tags within a series of predefined windows of equal lengths along different chromosomes, more robust and statistically significant results may be obtained.” *Id.* at col. 4, ll. 62–67.

The ’076 patent also provides examples that describe “direct sequencing of cell-free DNA from plasma of pregnant women with high throughput shotgun sequencing technology.” *Id.* at col. 20, ll. 30–32. The sequences are mapped to specific chromosomal regions, allowing for the measurement of over- and under-representation of chromosomes from an aneuploid fetus. *Id.* at col. 20, ll. 34–36.

The ’076 patent discloses further that “[a]nother method for increasing sensitivity to fetal DNA is to focus on certain regions within the human genome,” such as by using sequencing methods that select sequences that map to a chromosome of interest *a priori*. *Id.* at col. 13, ll. 53–56. An area of focus may be a partial chromosome deletion, such as 22q11 deletion syndrome. *Id.* at col. 13, ll. 57–59. Sequence-based methods that may be used to sequence selected subsequences include sequencing by array, as well

as using capture beads with specific genomic sequences used as capture probes. *Id.* at col. 13, l. 65–col. 14, l. 1.

As taught by the '076 patent:

> The subsequencing method is in one aspect contrary to conventional massively parallel sequencing methodologies, which seek to obtain all of the sequence information in a sample. This alternative method selectively ignores certain sequence information by using a sequencing method which selectively captures sample molecules containing certain predefined sequences. One may also use the sequencing steps exactly as exemplified, but in mapping the sequence fragments obtained, give greater weight to sequences which map to areas known to be more reliable in their coverage, such as exons. Otherwise, the method proceeds as described below, where one obtains a large number of sequence reads from one or more reference chromosomes, which are compared to a large number of reads obtained from a chromosome of interest, after accounting for variations arising from chromosomal length, G/C content, repeat sequences and the like.

Id. at col. 14, ll. 21–39.

D. Illustrative Claims

Claim 1 is the only independent challenged claim. Claims 1–3 and 9 are illustrative of the disclosed invention, and are reproduced below (emphases added):

> **1.** A method of testing for an abnormal distribution of a chromosome in a sample comprising a mixture of maternal and fetal DNA, comprising the steps of:
>
> (a) obtaining maternal and fetal DNA from said sample;
>
> (b) *sequencing predefined subsequences* of the maternal and fetal DNA to obtain a plurality of sequence tags aligning to the predefined subsequences, wherein said sequence tags are of

sufficient length to be assigned to a specific predefined subsequence, wherein the predefined subsequences are from a plurality of different chromosomes, and wherein said plurality of different chromosomes comprise at least one first chromosome suspected of having an abnormal distribution in said sample and at least one second chromosome presumed to be normally distributed in said sample;

(c) assigning the plurality of sequence tags to their corresponding *predetermined subsequences*;

(d) determining a number of sequence tags aligning to the predetermined subsequences of said first chromosome and a number of sequence tags to the predetermined subsequences of the second chromosome; and

(e) comparing the numbers from step (d) to determine the presence or absence of an abnormal distribution of said first chromosome.

2. The method of claim 1 wherein the sample is a maternal serum or plasma sample, wherein the abnormal distribution of said first chromosome is a fetal aneuploidy, and wherein said second chromosome is a euploid chromosome.

3. The method of claim 2 wherein the sequencing comprises *massively parallel sequencing* of the predefined subsequences.

9. The method of claim 2 wherein said sequencing comprises selectively sequencing nucleic acid molecules comprising the predefined sequences.

Ex. 1001, col. 35, ll. 10–41, col. 36, ll. 8–10.

E. Instituted Challenges

Claims	Basis	References
1–5, 7–9, 12, and 13	§ 102(e)	Lo[1]
10 and 11	§ 103(a)	Lo and Brenner[2]
1–5 and 7–13	§ 103(a)	Quake[3] and Kapur[4]
6	§ 103(a)	Lo and Li;[5] and Quake, Kapur, and Li

II. ANALYSIS

A. Claim Construction

In an *inter partes* review, claim terms in an unexpired patent are interpreted according to their broadest reasonable construction in light of the specification of the patent in which they appear. 37 C.F.R. § 42.100(b); Office Patent Trial Practice Guide, 77 Fed. Reg. 48,756, 48,766 (Aug. 14, 2012). Claim terms also are given their ordinary and customary meaning, as would be understood by one of ordinary skill in the art in the context of the entire disclosure. *In re Translogic Tech., Inc.*, 504 F.3d 1249, 1257 (Fed. Cir. 2007). If an inventor acts as his or her own lexicographer,

[1] Lo et al., Pub. No. US 2009/0029377, published Jan. 29, 2009 (Ex. 1004, "Lo").
[2] Brenner, Pub. No. US 2006/0177832 A1, published Aug. 10, 2006 (Ex. 1003, "Brenner").
[3] Quake et al., Pub. No. US 2007/0202525 A1, published Aug. 30, 2007 (Ex. 1006, "Quake").
[4] Kapur et al., Pub. No. US 2008/0138809 A1, published Jun. 12, 2008 (Ex. 1005, "Kapur").
[5] Heng Li et al., *Mapping Short DNA Sequencing Reads and Calling Variants Using Mapping Quality Scores*, 18 GENOME RESEARCH 1851–1858 (2008) (Ex. 1014, "Li").

the definition must be set forth in the specification with reasonable clarity, deliberateness, and precision. *Renishaw PLC v. Marposs Societa' per Azioni*, 158 F.3d 1243, 1249 (Fed. Cir. 1998). "[A] claim construction that excludes a preferred embodiment . . . is rarely, if ever correct and would require highly persuasive evidentiary support." *Epos Tech. Ltd. v. Pegasus Tech. Ltd.*, 766 F.3d 1338, 1347 (Fed. Cir. 2014) (alteration in original) (internal citation and quotations omitted). Moreover, "[u]nder the doctrine of claim differentiation, dependent claims are presumed to be of narrower scope than the independent claims from which they depend." *AK Steel Corp. v. Sollac and Ugine*, 344 F.3d 1234, 1242 (Fed. Cir. 2003) (internal citation omitted).

1. "sequence tag"

The '076 patent defines "sequence tag" as a "DNA sequence of sufficient length that it may be assigned specifically to one of chromosomes 1–22, X or Y." Ex. 1001, col. 8, ll. 57–59. We adopt that construction.

2. "massively parallel sequencing"

Neither party explicitly requests a construction of this term, which is found in dependent claims 3 and 4, but we set forth the definition provided by the '076 patent, as it informs our construction of other portions of the claim whose construction is at issue. Specifically, the '076 patent defines "massively parallel sequencing" as

> techniques for sequencing millions of fragments of nucleic acids, e.g., using attachment of randomly fragmented genomic DNA to a planar, optically transparent surface and solid phase amplification to create a high density sequencing flow cell with millions of clusters, each containing ~1,000 copies of template per sq. cm. These templates are sequenced using four-color DNA sequencing-by-synthesis technology.

Ex. 1001, col. 9, ll. 22–29.

3. "sequencing predefined subsequences"

In the Institution Decision, we construed this term as not limited to any particular sequencing technique, and thus encompassing the use of random shotgun sequencing. Dec. Inst. 7–10.

Patent Owner contends that the method claimed by the '076 patent is limited to "targeted sequencing, *i.e.*, only certain predefined sequences are actually sequenced," which, Patent Owner asserts, excludes the use of random shotgun sequencing. PO Resp. 7. In particular, Patent Owner argues that the term "predefined" in the claim excludes random sequencing. *Id.* at 8. According to Patent Owner, although the Specification includes embodiments to both random and targeted sequencing, the ordinary artisan would understand that the use of the term "predefined" refers to targeted sequencing. *Id.* at 10–12.

Relying on the Declaration of Dr. J. Chris Detter, Patent Owner asserts that "a person of ordinary skill in the art would understand that the term 'predefined' refers to preselecting the nucleic acids to be sequenced prior to sequencing them." *Id.* at 8–9 (citing Ex. 2008 ¶ 48). Patent Owner argues further that step 1(b) of claim 1 also supports its construction, because the sequence tags are assigned only to predefined subsequences, and thus sequence tags that cannot be assigned to the predefined sequences are not produced. *Id.* at 9. According to Patent Owner, steps 1(c) and 1(d) of claim 1 also support its construction. *Id.* at 10. Specifically, Patent Owner argues that the use of the term "subsequence," as well as the term "predefined," "confirms that less than all of the chromosomes of interest are

being sequenced in the claimed method, consistent with targeted sequencing." *Id*.

Petitioner responds that Patent Owner's construction excludes the preferred and exemplified embodiments of the '076 patent, all of which involve the use of random shotgun sequencing. Reply 2.

We have considered Patent Owner's contentions carefully, as well as the evidence cited by Patent Owner, and we decline to adopt its proffered construction of limiting "sequencing predefined subsequences" to targeted sequencing. As noted by Petitioner, the term "targeted sequencing" nowhere appears in the Specification of the '076 patent. *Id.* at 1. Moreover, claim terms are not construed in isolation. Rather, claim terms should be construed in the context of the claim as a whole, in light of the teachings of the Specification. *See*, *e.g.*, *Hockerson-Halberstadt, Inc. v. Converse Inc.*, 183 F.3d 1369, 1374 (Fed. Cir. 1999) ("Proper claim construction . . . demands interpretation of the entire claim in context, not a single element in isolation."). We find that the rest of claim 1, the dependent claims, and the Specification support our construction.

The portion of step (b) of claim 1 that includes the disputed phrase recites (emphasis added) "*sequencing predefined subsequences* of the maternal and fetal DNA to obtain a plurality of sequence tags aligning to the predefined subsequences." Thus, the claim language associates "sequencing predefined sequences" with obtaining "sequence tags." The Specification of the '076 patent discusses sequence tags in the context of shotgun sequencing. *See*, *e.g.*, Ex. 1001, col. 14, ll. 56–66. As Petitioner notes, there is no discussion in the Specification of the term "targeted sequencing." Reply 1. Instead, the '076 patent also discloses the use of a large number of

windows of defined length created along the chromosome, such that the windows cover each chromosome in question, except for the non-informative regions of the chromosome, such as centromere and repetitive regions, may be omitted. *Id.* at col. 4, ll. 56–62. Thus, "[v]arious average numbers, i.e., median values, are calculated for different windows and compared. By counting sequence tags within a series of *predefined* windows of equal lengths along different chromosomes, more robust and statistically significant results may be obtained." *Id.* at col. 4, ll. 62–67 (emphasis added). As taught by the '076 patent, "[e]ach autosome (chr. 1–22) is computationally segmented into contiguous, non-overlapping windows," although sliding windows could also be used. *Id.* at col. 5, ll. 4–9. In addition, as noted above, the '076 patent also provides examples that describe "direct sequencing of cell-free DNA from plasma of pregnant women with high throughput shotgun sequencing technology," wherein sequences were mapped to specific chromosomal regions, allowing for the measurement of over- and under-representation of chromosomes from an aneuploid fetus. *Id.* at col. 20, ll. 30–36. Thus, the Specification does not disclose sequencing *only* the defined sequences as Patent Owner would have us construe this phrase, but instead, it discloses sequencing the predefined sequences along with other sequences, and then using various techniques to locate the predefined sequences in the material that has been sequenced.

Thus, we conclude that the broadest reasonable interpretation of the phrase "sequencing predefined subsequences" is that the subsequences may be predefined through comparison with the predefined windows that are created along the length of the chromosome. The predefined subsequences may walk along the entire length of the chromosome. Thus, although one

predefined subsequence would not include the entire chromosomal sequence, two or more subsequences may include the entire length of the chromosomal sequence. That interpretation encompasses the preferred embodiment of the '076 patent, whereas Patent Owner's proffered construction limiting the method of claim 1 to "targeted" sequencing, as Patent Owner defines that term, would not

Steps (c) and (d) of claim 1 are not inconsistent with our construction. Step (c) requires "assigning the plurality of sequence tags to their corresponding *predetermined subsequences*" (emphasis added), which would encompass matching the sequence tag to its corresponding predefined window. Step (d) requires "determining a number of sequence tags aligning to the predetermined subsequences of said first chromosome and a number of sequence tags to the predetermined subsequences of the second chromosome," which encompasses counting the number of sequence tags that match the corresponding predefined window.

Moreover, the doctrine of claim differentiation supports our construction. Claim 9 requires that "said sequencing comprises selectively sequencing nucleic acid molecules comprising the predefined sequences." Claim 9 depends from claim 2, which depends from claim 1. The claim 9 limitation—that the sequencing step is limited to sequencing only the predefined sequences—further supports our interpretation that "sequencing predetermined sequences" is not limited to sequencing only the predefined sequences, but encompasses sequencing sequences in addition to the predefined sequences. Although we recognize that the doctrine of claim differentiation may be more of a "rebuttable presumption" than a "doctrine," *Retractable Technologies, Inc. v. Becton, Dickinson & Co.*, 653 F.3d 1296,

1305 (Fed. Cir. 2011), Patent Owner has not provided evidence or argument sufficient to rebut the presumption. *See also Seachange Intern., Inc. v. C-COR, Inc.*, 413 F.3d 1361, 1368-69 (noting that the "doctrine is at its strongest 'where the limitation sought to be "read into" an independent claim already appears in a dependent claim'").

Thus, consistent with the Institution Decision, we decline to limit "sequencing predefined sequences" to any particular sequencing method, but construe the term as encompassing sequencing methods such as random shotgun sequencing, as well as sequencing by hybridization. Dec. Inst. 7–10; *see also* Ex. 1041, 32–34 (District Court claim construction order noting that claim 1 is not limited to selectively capturing sample molecules, that is, molecular preselection).

In sum, we construe "sequencing predefined subsequences" as not limited to targeted sequencing, wherein the sequences are molecularly preselected, such as by hybridization; but as also encompassing informationally predefining the subsequences, such as through the use of the predefined windows taught by the '076 patent.

4. "predetermined subsequences"

In the Institution Decision, we construed "predetermined subsequences" as "reference sequence information." Dec. Inst. 10–11. Patent Owner contends that step (d) of claim 1 "states that the 'predetermined subsequences' are 'subsequences of said first chromosome' and subsequences of the second chromosome.'" PO Resp. 15. Thus, Patent Owner argues that "the predetermined subsequences are not just any reference sequence information, but rather are reference sequence information that represent less than all of a chromosome." *Id.* Specifically,

step (d) of claim 1 recites "determining a number of sequence tags aligning to the predetermined subsequences of said first chromosome and a number of sequence tags to the predetermined subsequences of the second chromosome." Given the explicit language of claim 1 that the sequence tags are aligned to a "predetermined subsequence" of a chromosome, to read that limitation as encompassing the entire chromosome would lead the limitation of "subsequence" out of the claim. Thus, we adopt Patent Owner's proposed construction: That is, we construe "predetermined subsequences" as "reference sequence information that represents less than all of a chromosome." Similar to our construction of "sequencing predefined subsequences," however, although one predetermined subsequence would not include the entire chromosomal sequence, two or more predetermined subsequences may include the entire length of the chromosomal sequence.

5. "polymorphism-independent"

Although the term "polymorphism-independent" does not appear in claim 1, Patent Owner argues that the claims of the '076 patent should be construed "as being directed to polymorphism-independent methods of detecting fetal aneuploidy." PO Resp. 15. Patent Owner contends that the '076 patent "repeatedly makes reference to the fact that the invention is polymorphism-independent." *Id.* Patent Owner does not point us to any claim language, however, that would limit the claim only to detecting fetal aneuploidies that are polymorphism-independent. Moreover, Patent Owner has not explained how the references to "polymorphism-independent" in the specification amount to a "clear disclaimer" of polymorphism dependent methods. *In re Am. Acad. Of Sci. Tech. Ctr.*, 367 F.3d 1359, 1369 (Fed. Cir. 2004). Thus, we determine that the broadest reasonable interpretation of the

method of claim 1 is that it encompasses methods of testing for both polymorphism dependent, and polymorphism-independent, abnormal distributions of a chromosome.

B. Patentability

To prevail on its challenges to the patentability of claims, Petitioner must prove unpatentability by a preponderance of the evidence. 35 U.S.C. § 316(e); 37 C.F.R. § 42.1(d).

1. Principles of Law

a. Anticipation

In order for a prior art reference to serve as an anticipatory reference, it must disclose every limitation of the claimed invention, either explicitly or inherently. *In re Schreiber*, 128 F.3d 1473, 1477 (Fed. Cir. 1997). We must analyze prior art references as a skilled artisan would. *See Scripps Clinic & Res. Found. v. Genentech, Inc.*, 927 F.2d 1565, 1576 (Fed. Cir. 1991), *overruled on other grounds by Abbott Labs. v. Sandoz, Inc.*, 566 F.3d 1282 (Fed. Cir. 2009) (to anticipate, "[t]here must be no difference between the claimed invention and the reference disclosure, as viewed by a person of ordinary skill in the field of the invention").

b. Obviousness

A claim is unpatentable under 35 U.S.C. § 103(a) if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. *KSR Int'l Co. v. Teleflex Inc.*, 550 U.S. 398, 406 (2007). The question of obviousness is resolved on the basis of underlying

factual determinations including: (1) the scope and content of the prior art; (2) any differences between the claimed subject matter and the prior art; (3) the level of ordinary skill in the art; and (4) objective evidence of nonobviousness. *Graham v. John Deere Co.*, 383 U.S. 1, 17–18 (1966). An invention "composed of several elements is not proved obvious merely by demonstrating that each of its elements was, independently, known in the prior art." *KSR*, 550 U.S. at 418. Moreover, a determination of unpatentability on the ground of obviousness must include "articulated reasoning with some rational underpinning to support the legal conclusion of obviousness." *In re Kahn*, 441 F.3d 977, 988 (Fed. Cir. 2006). The obviousness analysis "should be made explicit" and it "can be important to identify a reason that would have prompted a person of ordinary skill in the relevant field to combine the elements in the way the claimed new invention does." *KSR*, 550 U.S. at 418.

2. Patent Owner's Contention that Petitioner's Arguments are Legally Deficient

Patent Owner contends that the expert testimony relied upon by Petitioner "is legally deficient and does not reflect a proper anticipation or obviousness analysis." PO Resp. 16–17. According to Patent Owner, both of Petitioner's experts, Dr. Cynthia Casson Morton and Dr. Robert Nussbaum, "acknowledged that they applied an incorrect and overly broad understanding of the legal standard for anticipation," and Dr. Morton could not "accurately identify a distinction between obviousness and anticipation." *Id.* Patent Owner argues further that the testimony of Dr. Morton and Dr. Nussbaum is unreliable. PO Resp. 18–19. For example, Patent Owner argues that Dr. Nussbaum admitted in his deposition that he had not read the entirety of the '076 patent, and Dr. Morton admitted that

she did not review all of the materials cited in her Declaration. *Id.* at 18-19 (citing Ex. 1038, 9:23–24; Ex. 1037, 37:3–6). We note, however, that it is within our discretion to assign the appropriate weight to be accorded to the testimonial evidence of Dr. Morton and Dr. Naussbaum. Thus, we decline to dismiss testimony of Petitioner's experts out-of-hand and will give it the weight we find to be appropriate.

3. Anticipation under 35 U.S.C. § 102(e) of claims 1–5, 7–9, 12, and 13 by Lo

Petitioner contends that Lo anticipates the challenged claims to the extent that they encompass shotgun sequencing, and also anticipates the claims to the extent they encompass sequencing of selected targeted subsequences. Pet. 20. Petitioner also sets forth a claim chart demonstrating where each element of the claims is taught by the reference, and relies, initially, on the Declaration of Dr. Morton (Ex. 1008), as well as the Declaration of Dr. Nussbaum (Ex. 1009). *Id.* at 21–27; *see also* Dec. Inst. 11–16 (applying the teaching of Lo to the challenged claims). Patent Owner disagrees with Petitioner's assertions (PO Resp. 4–55), and relies on the Declaration of Dr. Detter (Ex. 2008) as evidence that Lo does not anticipate the challenged claims.

a. Lo (Ex. 1004)

Lo is drawn to "the diagnostic testing of fetal chromosomal aneuploidy by determining imbalances between different nucleic acid sequences, and more particularly to the identification of trisomy 21 (Down syndrome) and other chromosomal aneuploidies via testing a maternal sample (e.g.[,] blood)." Ex. 1004 ¶ 3; *see also* ¶ 46 (defining "chromosomal aneuploidy" as "a variation in the quantitative amount of a chromosome from that of a diploid genome"). According to Lo, a number of sequences

are randomly sequenced, wherein the number is based on the desired accuracy. *Id.* ¶ 18. Lo defines "random sequencing" as

> sequencing whereby the nucleic acid fragments sequenced have not been specifically identified or targeted before the sequencing procedure. Sequence-specific primers to target specific gene loci are not required. The pools of nucleic acids sequenced vary from sample to sample and even from analysis to analysis for the same sample. The identities of the sequenced nucleic acids are only revealed from the sequencing output generated.

Id. ¶ 47. Lo notes, however, that "the random sequencing may be preceded by procedures to enrich a biological sample with particular populations of nucleic acid molecules sharing certain common features." *Id.*

Lo teaches that a biological sample, such as plasma or serum, is obtained from a pregnant female, wherein the sample contains nucleic acid fragments from both the fetus and the mother. *Id.* ¶ 54. The nucleic acid fragments may then be sequenced, for example, by using massively parallel sequencing methods. *Id.* ¶¶ 55–56. Bioinformatics analysis may then be used to identify those sequence reads which map to a chromosome of interest. *Id.* ¶¶ 57–58; *see also id.* ¶ 70 (noting that sequencing from "Illumina Genome Analyzer" results in "short sequence tags," which "were aligned to the human reference genome sequence[,] and the chromosomal original was noted"). A second amount of one or more of a second chromosome is also determined. *Id.* ¶ 59. By taking into account the relative amount of the first chromosome to the second chromosome, a normalized frequency is obtained, which allows for the detection of an aneuploidy, such as a trisomy. *Id.* ¶ 69. Lo teaches generating sequence tags, which may be aligned to each chromosome, and compared to a

reference chromosome, to allow for the identification of chromosomal gains or losses. *Id.* ¶ 70.

Lo teaches that "the number of aligned sequenced tags were counted and sorted according to chromosomal location." *Id.* ¶ 71. As taught by Lo, "[g]ains or losses of chromosomal regions or whole chromosomes were determined by comparing the tag counts with the expected chromosome size in the reference genome or that of a non-disease representative specimen." *Id.*

Lo teaches another embodiment, wherein:

> the fraction of the nucleic acid pool that is sequenced in a run is further sub-selected prior to sequencing. For example, hybridization based techniques such as oligonucleotide array could be used to first sub-select for nucleic acid sequences from certain chromosomes, e.g.[,] a potentially aneuploid chromosome and other chromosome(s) not involved in the aneuploidy tested. Another example is that a certain sub-population of nucleic acid sequences from the sample pool is sub-selected or enriched prior to sequencing.

Id. ¶ 72.

In particular, Lo teaches that "sequences originating from a potentially aneuploid chromosome and one or more chromosomes not involved in the aneuploidy could be enriched by hybridization techniques for example onto oligonucelotide microarrays," which sequences would then be subject to random sequencing. *Id.* ¶ 79. Lo teaches further that one aspect of this massively parallel sequencing approach is that representative data from all of the chromosomes may be generated at the same time. *Id.* ¶ 80. Lo explains that although the sequencing is done at random, a database search may be performed to identify the chromosome from which a particular fragment originates. *Id.*

b. Analysis

Lo teaches a method of testing for an abnormal distribution of a chromosome, using genomic material obtained from maternal blood. Lo ¶ 3. Thus, Lo is drawn to a "method of testing for an abnormal distribution of a chromosome in a sample comprising a mixture of maternal and fetal DNA," as well as the step of "obtaining maternal and fetal DNA from said sample" as required by challenged claim 1.

Lo teaches also that the DNA fragments obtained from the sample may then be sequenced, for example, by using massively parallel sequencing methods. *Id.* ¶¶ 55–56. As taught by Lo, sequence tags are generated, aligned to each chromosome, and tags identified as originating from a potentially aneuploid chromosome are compared quantitatively to tags originating from a reference chromosome, to allow for the identification of chromosomal gains or losses. *Id.* ¶ 70. That falls within the scope of the limitation of step (b) of claim 1 of "sequencing predefined subsequences of the maternal and fetal DNA to obtain a plurality of sequence tags aligning to the predefined subsequences, wherein said sequence tags are of sufficient length to be assigned to a specific predefined subsequence." That is, as construed above, the sequencing step is not limited to sequencing only the predefined subsequences, but encompasses sequencing other portions of the genome. And, as Lo teaches that the sequence tags are aligned to a chromosome to allow for the identification of chromosomal gains or losses (*id.* ¶ 70), the tags generated must necessarily be of sufficient length to be assigned to a specific predefined subsequence. *See, e.g.*, Ex. 1008 ¶ 60 (noting that although Lo "does not describe specifically that the sequence tags were of sufficient length to be assigned to a specific region of the

genome, one skilled in the art would understand this to be the case, because the sequence tags used to identify chromosomal origin must have been of sufficient length to allow assignment to a particular genomic region").

Moreover, to the extent that the claim encompasses preselecting sequences, Lo teaches that a fraction of the nucleic acid pool may be further sub-selected prior to sequencing. Ex. 1004 ¶ 72. As taught by Lo, hybridization based techniques, such as the use of an oligonucleotide array, may be used to first sub-select for nucleic acid sequences from certain chromosomes, such as a potentially aneuploid chromosome, as well as a second chromosome not involved in the aneuploidy being tested. *Id.* Thus, although claim 1 does not specify a pre-selection step, Lo teaches a pre-selection step, wherein only certain subsequences of the total fraction of genomic material obtained from the maternal sample is sequenced, wherein the subsequences are selected for using a hybridization reaction, *e.g.*, such as hybridization to a DNA array.

In addition, Lo teaches that the sequencing data can be used to determine an amount of a "first chromosome," such as the chromosome being tested for aneuploidy, as well as one or more second chromosomes in the sample. *Id.* ¶ 58. As taught by Lo, gains or losses of chromosomal regions or whole chromosomes may then be determined by comparing the tag counts with the expected chromosome size in the reference genome to that of the reference chromosome. *Id.* ¶ 71. Thus, Lo teaches the limitation of step (b) of claim 1: "the predefined subsequences are from a plurality of different chromosomes, and wherein said plurality of different chromosomes comprise at least one first chromosome suspected of having an abnormal

distribution in said sample and at least one second chromosome presumed to be normally distributed in said sample."

Lo teaches that the sequence tags that are generated are aligned to the human reference genome sequence in order to determine their chromosomal origin (*id.* ¶ 70), and thus discloses a step of "assigning the plurality of sequence tags to their corresponding predetermined subsequences," as set forth in step (c) of claim 1. Although Lo states that the sequences are aligned with the reference genome sequence to determine their chromosomal origin, the sequence tags are nucleic acid fragments obtained from the maternal sample. Ex. 1004 ¶ 54. Thus, as the nucleic sample obtained from the pregnant female only contains chromosomal fragments, the fragments necessarily can only be aligned to the subsequence of the chromosome that corresponds to the sequence of the fragment.

As for step (d) of claim 1, which requires "determining a number of sequence tags aligning to the predetermined subsequences of said first chromosome and a number of sequence tags to the predetermined subsequences of the second chromosome," Lo discloses tabulating the total number of individual sequence tags aligned to each chromosome of the human reference genome sequence. Lo ¶ 70. Finally, Lo also teaches that "[g]ains or losses of chromosomal regions or whole chromosomes were determined by comparing the tag counts with the expected chromosome size in the reference genome or that of a non-disease representative specimen" (*id.* ¶ 71), and thus teaches step (e) of claim 1: "comparing the numbers from step (d) to determine the presence or absence of an abnormal distribution of said first chromosome."

Patent Owner argues that Lo cannot anticipate the challenged claims as "it does not teach at least the claimed element 'sequencing predefined subsequences.'" PO Resp. 20. Patent Owner asserts that Lo teaches only random sequencing, and that even Petitioner's expert, Dr. Morton, agrees that Lo does not teach how to pre-select nucleic acids for sequencing. *Id.* (citing Ex. 1037, 71–72). The random sequencing of Lo, Patent Owner argues, does not anticipate the claims when the claim term "predefined" is given the meaning of "targeted sequencing." *Id.* at 24. That is, the claim requires "the pre-selection of sequences prior to the sequencing step." *Id.* at 25.

Patent Owner asserts further that paragraph 72 of Lo does not teach "sequencing predefined subsequences," and that the Declarations of Dr. Morten and Dr. Nussbaum do not provide any analysis as to why that paragraph of Lo teaches that limitation. *Id.* at 20. According to Patent Owner, Lo at paragraph 72 discloses that hybridization is used to select the entire chromosome for sequencing. *Id.* at 22 (citing Ex. 2008 ¶ 66).

Patent Owner contends further that we erred in instituting the challenge based on Lo. That is, in the Institution Decision, we construed "sequencing predefined subsequences" as requiring that the subsequences should uniquely map to a chromosome region of interest (Dec. Inst. 10), but Lo uses hybridization to select an entire chromosome for sequencing, and not just a selected subsequence or region of the chromosome. PO Resp. 21–22. Patent Owner contends that although Lo teaches that sequences from a potentially aneuploid chromosome, as well as one or more chromosomes not involved in the aneuploidy, could be enriched by hybridization techniques, and then subjected to random sequencing, "[a] person of ordinary skill in the

art would understand that enriching a pool of nucleic acids for sequences originating from a chromosome is not the same as sequencing only particular predefined subsequences of the chromosome." *Id.* at 23 (citing Ex. 1004 ¶ 79; Ex. 2008 ¶ 68). We disagree.

Patent Owner's arguments are premised primarily on a narrow construction of "sequencing predefined subsequences" as being drawn to "targeted sequencing," wherein "only certain predefined sequences are actually sequenced"—a construction we have declined to adopt, as discussed above. *See* section II.A.3.

Moreover, to the extent that the claim requires preselecting sequences for sequencing, we do not find convincing Patent Owner's argument that paragraph 72 of Lo does not teach a pre-selection step. Specifically, Patent Owner relies on the Declaration of Dr. Detter, who states that "using an array to select all fragments associated with an entire chromosome[ ] is not the same concept as predefining subsequences for sequencing as required by the claims of the Fan '076 patent." Ex. 2008 ¶ 66; *see also id.* ¶ 68 (noting that "a person of ordinary skill in the art would understand that enriching a pool of nucleic acids for sequences originating from a chromosome is not the same as sequencing only particular predefined subsequences of the chromosome as in a targeted sequencing approach").

As noted above, the Specification of the '076 patent nowhere uses the term "targeted sequencing." In addition, although claim 1 uses the term "predefined subsequences," neither the language of the claim, nor the remainder of the Specification, defines how the subsequence is predefined or predetermined. As discussed above in section II.A.3, although we construed a single subsequence as not encompassing an entire chromosomal sequence,

we noted that two or more subsequences can encompass the entire length of the chromosome.

As taught by Lo (¶ 72), and acknowledged in the Specification of the '076 patent (Ex. 1001, col. 4, ll. 41–44), the fetal nucleic acids present in maternal plasma are short fragments. Lo teaches the use of an *oligonucleotide* (i.e., a short nucleotide molecule) array to sub-select for sequences from certain chromosomes. Thus, although Lo may in fact be using the oligonucleotide array to sub-select for sequences along the entire length of a desired chromosome, the oligonucleotides that make up the array are selecting for subsequences of the chromosome, which subsequences may then be analyzed using massively parallel sequencing. Lo thus teaches sequencing of predefined subsequences of a chromosome.

Also, our finding that Lo anticipates the method of challenged claim 1 is not inconsistent with our construing "sequencing predefined subsequences" to mean "sequencing predefined nucleic acid molecules that uniquely map to a chromosome region of interest in a reference genome." Specifically, as discussed above, the fetal nucleic acids that are present in plasma are short fragments. Lo teaches that the short sequence tags are aligned to a human reference sequence, and that the chromosomal origin is noted. Lo ¶ 70. Thus, in the alignment, the sequence tags are necessarily aligned with a region of the longer chromosome, given that the sequence tags are short fragments derived from chromosomal DNA.

For similar reasons, Patent Owner contends also that Lo does not disclose step (d) of claim 1, as "Lo makes it clear that predetermined sequences are not used in its method." PO Resp. 28. Lo teaches that after massively parallel sequencing, the chromosomal location of the sequenced

tags was determined. Patent Owner argues that Petitioner does not explain how that "involves determining a number of sequence tags aligning to predetermined subsequences of chromosomes." *Id.* That is, Patent Owner argues, "the Lo Publication makes clear that 'massively parallel sequencing is not dependent on the detection or analysis of predetermined or a predefined set of DNA sequences.'" *Id.* at 29 (emphasis omitted) (citing Ex. 1004 ¶ 108).

Again, Patent Owner's contentions are based, in large part, on a narrow construction of the term "sequencing predefined subsequences," a construction we decline to adopt for the reasons set forth above. Moreover, to the extent that the claims requires sequencing predefined subsequences, wherein the sequences are molecularly selected, paragraph 72 of Lo discusses the use of an *oligonucleotide* array to sub-select for sequences from certain chromosomes. In addition, Lo teaches tabulating the total number of individual sequence tags aligned to each chromosome of the human reference genome sequence. *Id.* ¶ 70. Thus, when a potential aneuploid chromosome and a reference chromosome are preselected using a DNA array, only those fragments that hybridize to the array are sequenced and aligned with chromosomal sequences, i.e., the sequence of a potentially aneuploid chromosome, as well as the sequence of a selected reference chromosome. Ex. 1043 ¶ 15. That method would meet the limitation of "determining a number of sequence tags aligning to the predetermined subsequences" of challenged claim 1.

Patent Owner does not present separate argument as to claims 2, 5, 7–9, 12, and 13. As to claims 3 and 4, Patent Owner argues that "Lo does not

disclose the use of a massively parallel sequencing system that sequences predefined subsequences." PO Resp. 29–30.

Lo teaches that "the fraction of the nucleic acid pool that is sequenced in a run is further sub-selected *prior* to sequencing." Lo ¶ 72 (emphasis added). Thus, the sub-selection occurs prior to sequencing. Lo also teaches that nucleic acid fragments may be sequenced, for example, by using massively parallel sequencing methods. *Id.* ¶¶ 55–56. The ordinary artisan would understand that the sub-selection described by paragraph 72 of Lo could be performed before performing any of the sequencing methods disclosed by Lo, including massively parallel sequencing.

c. Conclusion

After considering Petitioner's and Patent Owner's positions, as well as their supporting evidence, we determine that Petitioner has shown by a preponderance of the evidence that claims 1, 3, and 4 are unpatentable under 35 U.S.C. § 102(e) as anticipated by Lo. In addition, we have reviewed Petitioner's position and evidence as to claims 2, 5, 7–9, 12, and 13, and determine that Petitioner has also shown by a preponderance of the evidence that those claims are unpatentable under 35 U.S.C. § 102(e) as anticipated by Lo.

4. Obviousness of Claims 10 and 11 over the Combination of Lo and Brenner (Ex. 1003)

Petitioner contends that claims 10 and 11 are rendered obvious by the combination of Lo and Brenner (Pet. 28–29). Patent Owner presents no evidence or argument demonstrating how Petitioner's contentions are incorrect. Upon review of claims 10 and 11, as well as the contentions and evidence relied upon by Petitioner, we determine that the preponderance of

the evidence of record demonstrates that those claims are rendered unpatentable by the combination of Lo and Brenner.

5. Obviousness of Claims 1–5 and 7–13 over the combination of Quake (Ex. 1006) and Kapur (Ex. 1005)

Petitioner contends that the combination of Quake and Kapur teaches all the limitations of the challenged claims (Pet. 29–39), and relies, initially, on the Declaration of Dr. Morton (Ex. 1008), as well as the Declaration of Dr. Nussbaum (Ex. 1009), for a rationale to combine those elements. Patent Owner disagrees with Petitioner's assertions (PO Resp. 30–60) and relies on the Declaration of Dr. Detter (Ex. 2008) as evidence that it would not have been obvious for one of ordinary skill in the art to combine the teachings of the references in the manner set forth by Petitioner.

a. Quake (Ex. 1006)

Quake is drawn to the use of "digital PCR" to detect fetal aneuploidies, such as Down's syndrome, which is a chromosomal trisomy. Ex. 1006 ¶ 9. In the method of Quake, a sample is obtained from the mother, wherein the sample is preferably maternal peripheral blood of blood plasma or serum, wherein the sample contains a mixture of maternal and fetal genetic material. *Id.* ¶ 26. The genetic material is then distributed into discrete samples, such that each sample does not contain, on average, more than one target sequence per sample. *Id.* ¶ 27. Quake teaches that "[t]he presence or absence of different target sequences in the discrete samples is detected; and the results are analyzed whereby the number of results from the discrete sample will provide data sufficient to obtain results distinguishing different target sequences." *Id.* In addition, Quake uses a

control sequence to detect an abnormal increase in the target sequence, such as a trisomy. *Id.* ¶ 54.

Quake teaches further the quantitative analysis of the detection of maternal and fetal nucleic acid target sequences, which may "include targets to different regions such as probes to a target on a chromosome suspected of being present in an abnormal copy number (trisomy) compared to a normal diploid chromosome, which is used as a control." *Id.* ¶ 61. Specifically, Quake teaches that detection may "be conveniently . . . carried out by a sequence specific probe," or by "directly sequencing a region of interest to determine if it is the target sequence of interest." Id. ¶ 84.

Quake teaches that the "method of differential detection of target sequences may involve direct sequencing of target sequences," wherein the sequencing may be of a single molecule, or of an amplified derivative of the target molecule. Id. ¶ 33. Additionally, Quake teaches the use of massively parallel sequencing to detect the target sequence. *Id.* ¶ 120. Quake describes the use of microfluidics to achieve the digital PCR conditions, but notes that the sample need not be separated into separate wells, but may be isolated on different beads, or by adhering to different areas of a substrate. *Id.* ¶¶ 112, 116. As taught by Quake, "[o]nly about 30 [base pairs]of random sequence information are needed to identify a sequence as belonging to a specific human chromosome," and teaches that software methods to identify a sequence to a known genome sequence are known. *Id.* ¶ 121.

b. Kapur (Ex. 1005)

Kapur discloses a method of enriching a rare cell population, such as fetal cells from a maternal peripheral blood sample, for the detection and diagnosis of fetal abnormalities. Ex. 1005 ¶ 5. Kapur teaches that genetic

conditions that can be determined include trisomy 13, trisomy 18, trisomy 21, Klinefelter Syndrome, dup(17)(11. 2p11. 2) syndrome, as well as Down syndrome. *Id.* ¶ 7. Specifically, according to Kapur, a sample from a pregnant female, such as a blood sample, is enriched for fetal cells. *Id.* ¶ 11. Genetic analysis is then performed on the cells, such as by SNP detection or RNA expression detection. *Id.* In some embodiments, the genetic analysis of SNP detection or RNA expression can be done using a microarray, wherein up to 100,000 SNPs may be detected in parallel, or 10,000 transcripts may be detected in parallel for detecting RNA expression. *Id.* ¶ 13. According to Kapur, the genetic analysis may be performed on DNA from chromosomes X, Y, 13, 18, or 21, and may also be performed on a control sample or reference sample, such as a maternal sample. *Id.* ¶ 114.

As taught by Kapur, the target cells can be selected and binned, resulting in the reduction of complexity and/or the total cell number of the output of the enriched cells. *Id.* ¶¶ 87–88. In order to analyze the genetic material, Kapur teaches that "target nucleic acids from a test sample are amplified and optionally results are compared with amplification of similar target nucleic acids from a non-rare cell population (reference sample)." *Id.* ¶ 109. According to Kapur, the nucleic acid of interest may also be preamplified, such as by amplification of outer primers in a nested PCR approach. *Id.* ¶ 111. The amplified nucleic acids may then be quantified, for example for determining gene or allele number, such as by using microarrays. *Id.* ¶ 112–113.

Kapur teaches:

> In some embodiments, analysis involves detecting one or more mutations or SNPs in DNA from e.g., enriched rare cells or enriched rare DNA. Such detection can be performed using,

> for example, DNA microarrays. Examples of DNA microarrays include those commercially available from Affymetrix, Inc. (Santa Clara, Calif.), including the Gene-Chip™Mapping Arrays including Mapping 100K Set, Mapping 10K 2. 0 Array, Mapping 10K Array, Mapping 500K Array Set, and GeneChip™ Human Mitochondrial Resequencing Array 2.0. The Mapping 10K array, Mapping 100K array set, and Mapping 500K array set analyze more than 10,000, 100,000 and 500,000 different human SNPs, respectively. . . . . In some embodiments, a microarray is used to detect at least 5, 10, 20, 50, 100, 200, 500, 1,000, 2,000, 5,000[,] 10,000, 20,000, 50,000, 1,00[0],000, 200,000, or 500, 000 different nucleic acid target(s) (e.g., SNPs, mutations or STRs) in a sample.

Id. ¶ 114. Kapur also notes that computer implemented methods for estimating copy number based on hybridization intensity are known. *Id.* ¶ 115.

Kapur provides an exemplary method in Figure 6, which is an overview of a process of using a SNP detection microarray. *Id.* ¶ 117. Figure 7 is an overview of another method of detecting mutations or SNPs using bead arrays. *Id.* ¶ 123.

c. Analysis

Petitioner contends that "[i]t would have been obvious to use the sequencing by array methods taught by *Kapur* in combination with analysis of targeted gene loci taught by *Quake* to identify fetal abnormalities." Pet. 30. Patent Owner responds that the combination of Quake and Kapur would not have rendered the method of the challenged claims obvious, as Kapur does not teach a method of sequencing by array, but in fact teaches a method of genotyping by single nucleotide polymorphism ("SNP") analysis via a hybridization array. PO Resp. 32, 44. Thus, Patent Owner contends,

the ordinary artisan would not have used the SNP detection array as taught by Kapur as the sequencing method of Quake. *Id.* at 51.

According to Patent Owner, in a method of sequencing by array, the probes on the array "will contain all or most of the possible nucleotide combinations within a given length," and the method is thus not sequence-dependent. *Id.* at 44; *see also id.* at 45–46 (describing a method of sequencing by hybridization). Arrays that are used for SNP detection, Patent Owner contends, are not the same as those used for sequencing by hybridization, as the SNP arrays do not contain every possible combination of nucleotides. *Id.* at 47–48; *see also id.* at 46–47 (describing a method of SNP detection). Patent Owner argues that the ordinary artisan also would have understood that sequencing by array, and the use of arrays to detect SNPs, are two separate fields. *Id.* at 48.

We agree with Patent Owner that, based on the record currently before us, Petitioner has not demonstrated that the ordinary artisan would have combined Quake and Kapur as set forth in the Petition (29–39) to arrive at the method of challenged claim 1.

In particular, Petitioner relies on Figures 6 and 7 of Kapur as teaching "a method for sequence determination of randomly generated DNA fragments using arrays having oligonucleotides of known sequence. Sequencing of a randomly generated genomic fragment via binding to a predefined sequence on an array aligns a random sequence to a probe indicative of a specific genomic region." Pet. 31. The evidence relied upon by Petitioner does not explain, however, why one would have a used a method for detecting SNPs as taught by Kapur to perform the sequencing of Quake. That is, what is lacking in the Petition and accompanying

Declarations is an "articulated reason[] with some rational underpinning to support the legal conclusion of obviousness." *Kahn*, 441 F.3d at 988.

Petitioner responds that the "sequencing by array technology of Balasubramanian (US 2003/0022207 A1, published Jan. 30, 2003, Ex. 1040) is cited in all of Kapur (Ex. 1005 ¶ 163), Quake (Ex. 1006 ¶ 120), *and* the '076 patent (Ex. 1001 at col. 10, lines 50–56)." Paper 34 ¶ 19. Figures 6 and 7 relied upon by Petitioner, however, are drawn to methods of detecting SNPs, and not to the sequencing array technology of Balasubramanian. In fact, Petitioner first relies on paragraph 163 of Kapur, which it contends cites the sequencing array technology of Balasubramanian, in its challenge of claim 3, which is drawn to the use of massively parallel sequencing. Petitioner cannot rely upon Balasubramanian in its Reply to make up for the deficiencies in its Petition. S*ee, e.g.*, 37 C.F.R. § 42.23(b) (noting that "[a]ll arguments for the relief requested in a motion must be made in the motion," and that a "reply may only respond to arguments raised in the corresponding opposition or patent owner response").

Petitioner contends further that "Dr. Detter conceded on cross-examination that sequencing **is** detection and Prof. Morton concurs with that view." Reply 14 (citing Ex. 2023, 59, ll. 20–23; Ex. 1043 ¶ 50). Specifically, in response to the question of whether you can detect nucleic acid by sequencing, Dr. Detter testified "[t]hat is what you detect by sequencing, is a nucleic acid." Ex. 2023, 59, ll. 20–23. We do not disagree with Petitioner that, by virtue of the ability of the nucleic acid to hybridize to a sequence on the SNP array, the ordinary artisan would have understood that if the sequence of the sequence on the array is known, one can determine the sequence of the nucleic acid that hybridized to it. This

reasoning does not explain, however, why the ordinary artisan would have used the arrays for detecting SNPs as taught by Kapur in the method of Quake.

Moreover, in its observations of the cross-examination of Dr. Morton, Patent Owner argues that "Dr. Morton testified that people of ordinary skill in the art would not use or understand the term 'sequencing' to refer to the use of SNP detection arrays," which is consistent with Dr. Nussbaum's testimony. Paper 31, 8 (citing Ex. 1051, 139–141; Ex. 1038, 17–18); *see also id.* at 10 (noting that Dr. Morton stated on cross-examination that SNP detection as shown in Figure 6 of Kapur is not a sequencing method, but a genotyping method (citing Ex. 1051, 143–145)).

Specifically, Dr. Morton testified that SNP detection is "in some ways . . . sequencing, but not the way we would typically think of determining perhaps a sequence." Ex. 1051, 139. According to Dr. Morton, it would be referred to as genotyping, noting, however, that the genotype is obtained from the sequence. *Id.* at 139. When asked whether "[u]sing these methods for genotyping would not be considered sequencing as you believe people skilled in the art use the term 'sequencing,'" Dr. Morton responded:

> I think using BeadChips and the Affy chips, people would typically refer to as genotyping and not sequencing. If I was going to tell somebody I was going to genotype a sample, they wouldn't necessarily -- if I was going to -- there wouldn't be a confusion between -- if I said I was going to sequence a sample, they'd think I'm going to get a different output than the genotyping, because the genotyping, you're looking for a specific SNP or to -- to classify that individual. But it is sequenced. It is nucleotide.

Id. at 141. Dr. Morton's testimony further supports our determination that the ordinary artisan would not have used the method of genotyping using SNP detection for the sequencing required by the method of Quake.

d. Conclusion

After considering Petitioner's and Patent Owner's positions, as well as their supporting evidence, we determine that Petitioner has not shown by a preponderance of the evidence that claims 1–5 and 7–13 are unpatentable under 35 U.S.C. § 103(a) as obvious over Quake and Kapur.

6. Obviousness of Claim 6 over the combination of Lo and Li (Ex. 1014), and Over Quake, Kapur, and Li

Petitioner contends that claim 6 is rendered obvious by the combination of Lo and Li, or the combination of Quake, Kapur, and Li (Pet. 58–59). Patent Owner presents no evidence or argument demonstrating how Petitioner's contentions are incorrect. Upon review of claim 6, as well as the contentions and evidence relied upon by Petitioner as to the combination of Lo and Li, we determine that the preponderance of the evidence of record demonstrates that the claim is rendered obvious by that combination.

As to the challenge based on the combination of Quake, Kapur, and Li, we have already determined that Petitioner has not established by a preponderance of the evidence that the combination of Quake and Kapur renders independent claim 1 obvious. For the same reasons, we conclude that Petitioner has not established by a preponderance of the evidence that the combination of Quake, Kapur, and Li renders claim 6, which is dependent on claim 1, obvious.

C. Patent Owner's Motion to Exclude Evidence (Paper 30)

Patent Owner asks us to exclude the Second Declaration of Dr. Morton (Ex. 1043), Exhibits 1044–1049, as well as the portions of Petitioner's Reply that rely on Exhibit 1043.

We relied only on paragraph 15 of Exhibit 1043, and did not rely on the remainder of that Declaration, nor did we rely on Exhibits 1044–1049 in making our final determination. We conclude, therefore, that it is unnecessary to consider Patent Owner's objections to the admissibility of those Exhibits, except to the extent that Patent Owner objects to the admissibility of paragraph 15 of Exhibit 1043.

As to paragraph 15 of Exhibit 1043, Patent Owner contends that the figure at the end of the paragraph contains an error. Paper 30, 7. That is, according to Patent Owner, "[w]hile most of the figure shows the life cycle of a single strand, the last element of the figure shows obtaining six sequence tags." *Id.* Dr. Morton admitted, however, "that using the sequencing techniques described in Lo, one would only get a single sequence tag from a single strand." *Id.* Patent Owner contends, therefore, that "the figure is both irrelevant under FRE 402 and misleading under FRE 403." *Id.*

Patent Owner's objections go more to the weight that paragraph 15 of Exhibit 1043 should be afforded, rather than to its admissibility. It is within our discretion to assign the appropriate weight to be accorded to Dr. Morton's testimonial evidence. The Board, sitting as a non-jury tribunal with administrative expertise, is well-positioned to determine and assign appropriate weight to evidence presented. *Gnosis S.P.A. v. S. Alabama Medical Science Foundation*, IPR2013-00118, slip op. at 43 (PTAB June 20,

2014) (Paper 64). *See also Donnelly Garment Co. v. NLRB*, 123 F.2d 215, 224 (8th Cir. 1941) ("One who is capable of ruling accurately upon the admissibility of evidence is equally capable of sifting it accurately after it has been received."). We thus decline to exclude paragraph 15 of Exhibit 1043.

III. CONCLUSION

Petitioner has shown by a preponderance of the evidence that claims 1–5, 7–9, 12, and 13 are unpatentable under 35 U.S.C. § 102(e) as anticipated by Lo;

Petitioner has shown by a preponderance of the evidence that claims 10 and 11 are unpatentable under 35 U.S.C. § 103(a) as obvious over the combination of Lo and Brenner;

Petitioner has shown by a preponderance of the evidence that claim 6 is unpatentable under 35 U.S.C. § 103(a) as obvious over the combination of Lo and Li;

Petitioner has not shown by a preponderance of the evidence that claims 1–5 and 7–13 are unpatentable under 35 U.S.C. § 103(a) over the combination of Quake and Kapur; and

Petitioner has not shown by a preponderance of the evidence that claim 6 is unpatentable under 35 U.S.C. § 103(a) over the combination of Quake, Kapur, and Li.

IV. ORDER

Accordingly, it is hereby:

ORDERED that Petitioner has shown by a preponderance of the evidence that claims 1–13 of the ’076 patent are unpatentable;

FURTHER ORDERED that Patent Owner’s Motion to Exclude Evidence is *denied* to the extent it seeks to exclude paragraph 15 of Exhibit 1043, and *dismissed* as moot as to the extent it seeks the exclusion of the remainder of Exhibit 1043, as well as Exhibits 1044–1049, as well as the portions of Petitioner’s Reply that rely on Exhibit 1043; and

FURTHER ORDERED that because this is a final written decision, parties to the proceeding seeking judicial review of the decision must comply with the notice and service requirements of 37 C.F.R. § 90.2.

IPR2013-00308
Patent 8,296,076 B2

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ADDENDUM 2

U.S. Patent No. 8,296,076 B2, Fan et al.

JA000040 – JA000087

2015-1413
The Board of Trustees of the Leland Stanford Junior University
v.
Ariosa Diagnostics, Inc.

US008296076B2

(12) **United States Patent**
Fan et al.

(10) **Patent No.: US 8,296,076 B2**
(45) **Date of Patent: Oct. 23, 2012**

(54) **NONINVASIVE DIAGNOSIS OF FETAL ANEUOPLOIDY BY SEQUENCING**

(75) Inventors: **Hei-Mun Christina Fan**, Fremont, CA (US); **Stephen R. Quake**, Stanford, CA (US)

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(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

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Related U.S. Application Data

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(60) Provisional application No. 61/098,758, filed on Sep. 20, 2008.

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(52) **U.S. Cl.** **702/20**

(58) **Field of Classification Search** 702/20, 702/182–185

See application file for complete search history.

(56) **References Cited**

U.S. PATENT DOCUMENTS

4,508,625 A 4/1985 Graham
4,675,286 A 6/1987 Calenoff
4,789,628 A 12/1988 Nayak
4,800,159 A 1/1989 Mullis et al.
4,971,904 A 11/1990 Luddy
4,977,078 A 12/1990 Niimura et al.
5,153,117 A 10/1992 Simons
5,215,926 A 6/1993 Etchells, III et al.
5,296,375 A 3/1994 Kricka et al.
5,300,779 A 4/1994 Hillman et al.
5,304,487 A 4/1994 Wilding et al.
(Continued)

FOREIGN PATENT DOCUMENTS

EP 0637996 B1 7/1997
(Continued)

OTHER PUBLICATIONS

U.S. Appl. No. 11/825,298, filed Jul. 5, 2007, Lopez et al.
(Continued)

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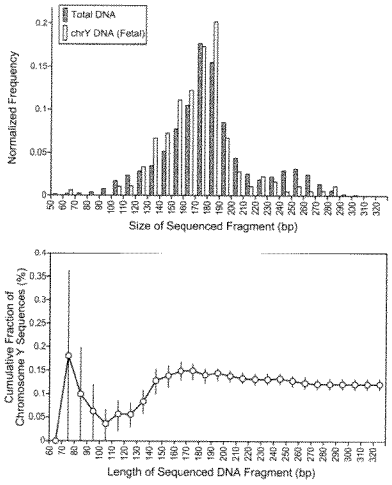
(57) **ABSTRACT**

Disclosed is a method to achieve digital quantification of DNA (i.e., counting differences between identical sequences) using direct shotgun sequencing followed by mapping to the chromosome of origin and enumeration of fragments per chromosome. The preferred method uses massively parallel sequencing, which can produce tens of millions of short sequence tags in a single run and enabling a sampling that can be statistically evaluated. By counting the number of sequence tags mapped to a predefined window in each chromosome, the over- or under-representation of any chromosome in maternal plasma DNA contributed by an aneuploid fetus can be detected. This method does not require the differentiation of fetal versus maternal DNA. The median count of autosomal values is used as a normalization constant to account for differences in total number of sequence tags is used for comparison between samples and between chromosomes.

15 Claims, 17 Drawing Sheets



U.S. PATENT DOCUMENTS

5,427,663	A	6/1995	Austin et al.
5,427,946	A	6/1995	Kricka et al.
5,432,054	A	7/1995	Saunders et al.
5,447,842	A	9/1995	Simons
5,486,335	A	1/1996	Wilding et al.
5,498,392	A	3/1996	Wilding et al.
5,529,903	A	6/1996	Kübler et al.
5,556,773	A	9/1996	Youmo
5,629,147	A	5/1997	Asgari et al.
5,639,669	A	6/1997	Ledley
5,641,628	A	6/1997	Bianchi
5,646,001	A	7/1997	Terstappen et al.
5,676,849	A	10/1997	Sammons et al.
5,707,799	A	1/1998	Hansmann et al.
5,709,943	A	1/1998	Coleman et al.
5,715,946	A	2/1998	Reichenbach
5,726,026	A	3/1998	Wilding et al.
5,750,339	A	5/1998	Smith
5,766,843	A	6/1998	Asgari et al.
5,770,029	A	6/1998	Nelson et al.
5,798,042	A	8/1998	Chu et al.
5,837,115	A	11/1998	Austin et al.
5,840,502	A	11/1998	Van Vlasselaer
5,842,787	A	12/1998	Koph-Sill et al.
5,858,649	A	1/1999	Asgari et al.
5,866,345	A	2/1999	Wilding et al.
5,879,883	A	3/1999	Benson et al.
5,891,651	A	4/1999	Roche et al.
5,928,880	A	7/1999	Wilding et al.
5,952,173	A	9/1999	Hansmann et al.
5,962,234	A	10/1999	Golbus
5,962,237	A	10/1999	Ts'o et al.
5,962,332	A	10/1999	Singer et al.
5,972,721	A	10/1999	Bruno et al.
5,993,665	A	11/1999	Terstappen et al.
5,994,057	A	11/1999	Mansfield
5,994,517	A	11/1999	Ts'o et al.
6,007,690	A	12/1999	Nelson et al.
6,008,007	A	12/1999	Fruehauf et al.
6,013,188	A	1/2000	Terstappen et al.
6,066,449	A	5/2000	Ditkoff et al.
6,074,827	A	6/2000	Nelson et al.
6,100,029	A	8/2000	Lapidus et al.
6,143,496	A	11/2000	Brown et al.
6,143,576	A	11/2000	Buechler
6,154,707	A	11/2000	Livak et al.
6,156,270	A	12/2000	Buechler
6,159,685	A	12/2000	Pinkel et al.
6,176,962	B1	1/2001	Soane et al.
6,184,043	B1	2/2001	Fodstad et al.
6,186,660	B1	2/2001	Koph-Sill et al.
6,197,523	B1	3/2001	Rimm et al.
6,200,765	B1	3/2001	Murphy et al.
6,210,891	B1	4/2001	Nyren et al.
6,214,558	B1	4/2001	Shuber et al.
6,235,474	B1	5/2001	Feinberg
6,258,540	B1	7/2001	Lo et al.
6,265,229	B1	7/2001	Fodstad et al.
6,300,077	B1	10/2001	Shuber et al.
6,344,326	B1	2/2002	Nelson et al.
6,361,958	B1	3/2002	Shieh et al.
6,365,362	B1	4/2002	Terstappen et al.
6,368,871	B1	4/2002	Christel et al.
6,376,181	B2	4/2002	Ramsey et al.
6,383,759	B1	5/2002	Murphy et al.
6,387,707	B1	5/2002	Seul et al.
6,391,559	B1	5/2002	Brown et al.
6,394,942	B2	5/2002	Moon et al.
6,399,364	B1	6/2002	Reeve et al.
6,432,630	B1	8/2002	Blankenstein
6,440,706	B1	8/2002	Vogelstein et al.
6,444,461	B1	9/2002	Knapp et al.
6,454,938	B2	9/2002	Moon et al.
6,479,299	B1	11/2002	Parce et al.
6,511,967	B1	1/2003	Weissleder et al.
6,517,234	B1	2/2003	Koph-Sill et al.
6,540,895	B1	4/2003	Spence et al.
6,576,478	B1	6/2003	Wagner et al.
6,582,904	B2	6/2003	Dahm
6,582,969	B1	6/2003	Wagner et al.
6,596,144	B1	7/2003	Regnier et al.
6,596,545	B1	7/2003	Wagner et al.
6,613,525	B2	9/2003	Nelson et al.
6,618,679	B2	9/2003	Loehriein et al.
6,632,619	B1	10/2003	Harrison et al.
6,632,652	B1	10/2003	Austin et al.
6,637,463	B1	10/2003	Lei et al.
6,645,731	B2	11/2003	Terstappen et al.
6,664,056	B2	12/2003	Lo et al.
6,664,104	B2	12/2003	Pourahmadi et al.
6,673,541	B1	1/2004	Klein et al.
6,674,525	B2	1/2004	Bardell et al.
6,685,841	B2	2/2004	Lopez et al.
6,689,615	B1	2/2004	Murto et al.
6,746,503	B1	6/2004	Benett et al.
6,753,147	B2	6/2004	Vogelstein et al.
6,783,928	B2	8/2004	Hvichia et al.
6,818,184	B2	11/2004	Fulwyler et al.
6,830,936	B2	12/2004	Anderson et al.
6,858,439	B1	2/2005	Xu et al.
6,875,619	B2	4/2005	Blackburn
6,893,881	B1	5/2005	Fodstad et al.
6,906,182	B2	6/2005	Ts'o et al.
6,911,345	B2	6/2005	Quake et al.
6,913,697	B2	7/2005	Lopez et al.
6,927,028	B2	8/2005	Dennis et al.
6,953,668	B1	10/2005	Israeli et al.
6,960,449	B2	11/2005	Wang et al.
7,115,709	B1	10/2006	Gray et al.
7,150,812	B2	12/2006	Huang et al.
7,171,975	B2	2/2007	Moon et al.
7,190,818	B2	3/2007	Ellis et al.
7,192,698	B1	3/2007	Kinch et al.
7,198,787	B2	4/2007	Fodstad et al.
7,208,275	B2	4/2007	Gocke et al.
7,212,660	B2	5/2007	Wetzel et al.
7,220,594	B2	5/2007	Foster et al.
7,227,002	B1	6/2007	Kufer et al.
7,229,838	B2	6/2007	Foster et al.
7,250,256	B2	7/2007	Reinhard et al.
7,252,976	B2	8/2007	Lin et al.
7,258,987	B2	8/2007	Lamorte et al.
7,262,177	B2	8/2007	Ts'o et al.
7,262,269	B2	8/2007	Lam et al.
7,264,972	B2	9/2007	Foster
7,272,252	B2	9/2007	De La Torre-Bueno et al.
7,276,170	B2	10/2007	Oakey et al.
7,332,277	B2	2/2008	Dhallan
7,442,506	B2	10/2008	Dhallan
7,476,363	B2	1/2009	Unger et al.
7,645,576	B2	1/2010	Lo et al.
7,655,399	B2	2/2010	Cantor et al.
2001/0007749	A1	7/2001	Feinberg
2001/0051341	A1	12/2001	Lo et al.
2001/0053958	A1	12/2001	Ried et al.
2002/0006621	A1	1/2002	Bianchi
2002/0009738	A1	1/2002	Houghton et al.
2002/0012930	A1	1/2002	Rothberg et al.
2002/0012931	A1	1/2002	Waldman et al.
2002/0016450	A1	2/2002	Laugharn et al.
2002/0019001	A1	2/2002	Light
2002/0028431	A1	3/2002	Julien
2002/0058332	A1	5/2002	Quake et al.
2002/0076825	A1	6/2002	Cheng et al.
2002/0086329	A1	7/2002	Shvets et al.
2002/0110835	A1	8/2002	Kumar
2002/0123078	A1	9/2002	Seul et al.
2002/0137088	A1	9/2002	Bianchi
2002/0164816	A1	11/2002	Quake
2002/0166760	A1	11/2002	Prentiss et al.
2002/0172987	A1	11/2002	Terstappen et al.
2003/0004402	A1	1/2003	Hitt et al.
2003/0017514	A1	1/2003	Pachmann et al.
2003/0022207	A1	1/2003	Balasubramanian
2003/0033091	A1	2/2003	Opalsky et al.
2003/0044388	A1	3/2003	Dennis et al.
2003/0072682	A1	4/2003	Kikinis

2003/0077292 A1 4/2003 Hanash et al.
2003/0119077 A1 6/2003 Ts'o et al.
2003/0119724 A1 6/2003 Ts'o et al.
2003/0129676 A1 7/2003 Terstappen et al.
2003/0153085 A1 8/2003 Leary et al.
2003/0159999 A1 8/2003 Oakey et al.
2003/0165852 A1 9/2003 Schueler et al.
2003/0170631 A1 9/2003 Houghton et al.
2003/0170703 A1 9/2003 Piper et al.
2003/0175990 A1 9/2003 Heyenga
2003/0186255 A1 10/2003 Williams et al.
2003/0190602 A1 10/2003 Pressman et al.
2003/0199685 A1 10/2003 Pressman et al.
2003/0204331 A1 10/2003 Whitney et al.
2003/0206901 A1 11/2003 Chen
2003/0232350 A1 12/2003 Afar et al.
2004/0005582 A1 1/2004 Shipwash
2004/0009471 A1 1/2004 Cao
2004/0018116 A1 1/2004 Desmond et al.
2004/0018509 A1 1/2004 Bianchi
2004/0043506 A1 3/2004 Haussecker et al.
2004/0048360 A1 3/2004 Wada et al.
2004/0053352 A1 3/2004 Ouyang et al.
2004/0072278 A1 4/2004 Chou et al.
2004/0096892 A1 5/2004 Wang et al.
2004/0137452 A1 7/2004 Levett et al.
2004/0137470 A1 7/2004 Dhallan
2004/0142463 A1 7/2004 Walker et al.
2004/0144651 A1 7/2004 Huang et al.
2004/0166555 A1 8/2004 Braff et al.
2004/0171091 A1 9/2004 Lesko et al.
2004/0185495 A1 9/2004 Schueler et al.
2004/0203037 A1 10/2004 Lo et al.
2004/0209299 A1 10/2004 Pinter et al.
2004/0214240 A1 10/2004 Cao
2004/0232074 A1 11/2004 Peters et al.
2004/0241707 A1 12/2004 Gao et al.
2005/0014208 A1 1/2005 Krehan et al.
2005/0019792 A1 1/2005 McBride et al.
2005/0037388 A1 2/2005 Antonarakis et al.
2005/0042623 A1 2/2005 Ault-Riche et al.
2005/0042685 A1 2/2005 Albert et al.
2005/0049793 A1 3/2005 Paterlini-Brechot
2005/0061962 A1 3/2005 Mueth et al.
2005/0118591 A1 6/2005 Tamak et al.
2005/0129581 A1 6/2005 McBride et al.
2005/0145496 A1 7/2005 Goodsaid et al.
2005/0147977 A1 7/2005 Koo et al.
2005/0153342 A1 7/2005 Chen
2005/0158754 A1 7/2005 Puffenberger et al.
2005/0164241 A1 7/2005 Hahn et al.
2005/0175996 A1 8/2005 Chen
2005/0181353 A1 8/2005 Rao et al.
2005/0181410 A1 8/2005 Shaffer et al.
2005/0181463 A1 8/2005 Rao et al.
2005/0196785 A1 9/2005 Quake et al.
2005/0207940 A1 9/2005 Butler et al.
2005/0211556 A1 9/2005 Childers et al.
2005/0214855 A1 9/2005 Wagner et al.
2005/0221341 A1 10/2005 Shimkets et al.
2005/0221373 A1 10/2005 Enzelberger et al.
2005/0239101 A1 10/2005 Sukumar et al.
2005/0244843 A1 11/2005 Chen et al.
2005/0250111 A1 11/2005 Xie et al.
2005/0250155 A1 11/2005 Lesko et al.
2005/0250199 A1 11/2005 Anderson et al.
2005/0252773 A1 11/2005 McBride et al.
2005/0255001 A1 11/2005 Padmanabhan et al.
2005/0262577 A1 11/2005 Guelly et al.
2005/0266433 A1 12/2005 Kapur et al.
2005/0272103 A1 12/2005 Chen
2005/0282196 A1 12/2005 Costa
2005/0282293 A1 12/2005 Cosmen et al.
2005/0287611 A1 12/2005 Nugent et al.
2006/0000772 A1 1/2006 Sano et al.
2006/0008807 A1 1/2006 O'Hara et al.
2006/0008824 A1 1/2006 Ronaghi et al.
2006/0024756 A1 2/2006 Tibbe et al.
2006/0046258 A1 3/2006 Lapidus et al.
2006/0051265 A1 3/2006 Mohamed et al.
2006/0051775 A1 3/2006 Bianchi et al.
2006/0060767 A1 3/2006 Wang et al.
2006/0072805 A1 4/2006 Tsipouras et al.
2006/0073125 A1 4/2006 Clarke et al.
2006/0094109 A1 5/2006 Trainer
2006/0121452 A1 6/2006 Dhallan
2006/0121624 A1 6/2006 Huang et al.
2006/0128006 A1 6/2006 Gerhardt et al.
2006/0134599 A1 6/2006 Toner et al.
2006/0160105 A1 7/2006 Dhallan
2006/0160150 A1 7/2006 Seilhamer et al.
2006/0160243 A1 7/2006 Tang et al.
2006/0183886 A1 8/2006 Ts'o et al.
2006/0205057 A1 9/2006 Wayner et al.
2006/0223178 A1 10/2006 Barber et al.
2006/0252054 A1 11/2006 Lin et al.
2006/0252061 A1 11/2006 Zabeau et al.
2006/0252068 A1 11/2006 Lo et al.
2006/0252071 A1 11/2006 Lo et al.
2006/0252087 A1 11/2006 Tang et al.
2007/0015171 A1 1/2007 Bianchi et al.
2007/0017633 A1 1/2007 Tonkovich et al.
2007/0026381 A1 2/2007 Huang et al.
2007/0026413 A1 2/2007 Toner et al.
2007/0026414 A1 2/2007 Fuchs et al.
2007/0026415 A1 2/2007 Fuchs et al.
2007/0026416 A1 2/2007 Fuchs
2007/0026417 A1 2/2007 Fuchs et al.
2007/0026418 A1 2/2007 Fuchs et al.
2007/0026419 A1 2/2007 Fuchs et al.
2007/0026469 A1 2/2007 Fuchs et al.
2007/0037172 A1 2/2007 Chiu et al.
2007/0037173 A1 2/2007 Allard et al.
2007/0037273 A1 2/2007 Shuler et al.
2007/0037275 A1 2/2007 Shuler et al.
2007/0042238 A1 2/2007 Kim et al.
2007/0042339 A1 2/2007 Toner et al.
2007/0042360 A1 2/2007 Afar et al.
2007/0042368 A1 2/2007 Zehentner-Wilkinson et al.
2007/0048750 A1 3/2007 Peck et al.
2007/0054268 A1 3/2007 Sutherland et al.
2007/0054287 A1 3/2007 Bloch
2007/0059680 A1 3/2007 Kapur et al.
2007/0059683 A1 3/2007 Barber et al.
2007/0059716 A1 3/2007 Balis et al.
2007/0059718 A1 3/2007 Toner et al.
2007/0059719 A1 3/2007 Grisham et al.
2007/0059737 A1 3/2007 Baker et al.
2007/0059774 A1 3/2007 Grisham et al.
2007/0059781 A1 3/2007 Kapur et al.
2007/0059785 A1 3/2007 Bacus et al.
2007/0065845 A1 3/2007 Baker et al.
2007/0065858 A1 3/2007 Haley
2007/0071762 A1 3/2007 Ts'o et al.
2007/0072228 A1 3/2007 Brauch
2007/0072290 A1 3/2007 Hvichia
2007/0077578 A1 4/2007 Penning et al.
2007/0092444 A1 4/2007 Benos et al.
2007/0092881 A1 4/2007 Ohnishi et al.
2007/0092917 A1 4/2007 Guyon
2007/0099207 A1 5/2007 Fuchs et al.
2007/0099219 A1 5/2007 Teverovskiy et al.
2007/0099289 A1 5/2007 Irimia et al.
2007/0105105 A1 5/2007 Clelland et al.
2007/0105133 A1 5/2007 Clark et al.
2007/0110773 A1 5/2007 Asina et al.
2007/0117158 A1 5/2007 Coumans et al.
2007/0122856 A1 5/2007 Georges et al.
2007/0122896 A1 5/2007 Shuler et al.
2007/0128655 A1 6/2007 Obata
2007/0131622 A1 6/2007 Oakey et al.
2007/0134658 A1 6/2007 Bohmer et al.
2007/0134713 A1 6/2007 Cao
2007/0135621 A1 6/2007 Bourel et al.
2007/0141587 A1 6/2007 Baker et al.
2007/0141588 A1 6/2007 Baker et al.
2007/0141717 A1 6/2007 Carpenter et al.
2007/0154928 A1 7/2007 Mack et al.

2007/0154960	A1	7/2007	Connelly et al.
2007/0160503	A1	7/2007	Sethu et al.
2007/0160974	A1	7/2007	Sidhu et al.
2007/0160984	A1	7/2007	Huang et al.
2007/0161064	A1	7/2007	Kinch et al.
2007/0166770	A1	7/2007	Hsieh et al.
2007/0170811	A1	7/2007	Rubel
2007/0172903	A1	7/2007	Toner et al.
2007/0178067	A1	8/2007	Maier et al.
2007/0178458	A1	8/2007	O'Brien et al.
2007/0187250	A1	8/2007	Huang et al.
2007/0196663	A1	8/2007	Schwartz et al.
2007/0196820	A1	8/2007	Kapur et al.
2007/0196840	A1	8/2007	Roca et al.
2007/0196869	A1	8/2007	Perez et al.
2007/0202106	A1	8/2007	Palucka et al.
2007/0202109	A1	8/2007	Nakamura et al.
2007/0202525	A1	8/2007	Quake et al.
2007/0202536	A1	8/2007	Yamanishi et al.
2007/0207351	A1	9/2007	Christensen et al.
2007/0207466	A1	9/2007	Cantor et al.
2007/0212689	A1	9/2007	Bianchi et al.
2007/0212698	A1	9/2007	Bendele et al.
2007/0212737	A1	9/2007	Clarke et al.
2007/0212738	A1	9/2007	Haley et al.
2007/0231851	A1	10/2007	Toner et al.
2007/0238105	A1	10/2007	Barrett et al.
2007/0259424	A1	11/2007	Toner et al.
2007/0264675	A1	11/2007	Toner et al.
2007/0275402	A1	11/2007	Lo et al.
2008/0020390	A1	1/2008	Mitchell et al.
2008/0023399	A1	1/2008	Inglis et al.
2008/0026390	A1	1/2008	Stoughton et al.
2008/0038733	A1	2/2008	Bischoff et al.
2008/0050739	A1	2/2008	Stoughton et al.
2008/0070792	A1	3/2008	Stoughton et al.
2008/0071076	A1	3/2008	Hahn et al.
2008/0090239	A1	4/2008	Shoemaker et al.
2008/0096216	A1	4/2008	Quake
2008/0096766	A1	4/2008	Lee
2008/0124721	A1	5/2008	Fuchs
2008/0138809	A1	6/2008	Kapur et al.
2008/0153090	A1	6/2008	Lo et al.
2008/0182261	A1	7/2008	Bianchi
2008/0193927	A1	8/2008	Mann et al.
2008/0213775	A1	9/2008	Brody et al.
2008/0220422	A1	9/2008	Shoemaker et al.
2008/0299562	A1	12/2008	Oeth et al.
2009/0029377	A1	1/2009	Lo et al.
2009/0087847	A1	4/2009	Lo et al.
2009/0170113	A1	7/2009	Quake et al.
2009/0170114	A1	7/2009	Quake et al.
2009/0215042	A1*	8/2009	Sella-Tavor et al. 435/6
2009/0280492	A1	11/2009	Stoughton et al.
2009/0291443	A1	11/2009	Stoughton et al.
2009/0307181	A1*	12/2009	Colby et al. 706/54
2010/0093835	A1*	4/2010	McSwiggen et al. ..... 514/44 A

FOREIGN PATENT DOCUMENTS

EP	0405972	B1	5/1999
EP	1262776	A2	12/2002
EP	0994963	B1	5/2003
EP	0970365	B1	10/2003
EP	783694	B1	11/2003
EP	1262776	A3	1/2004
EP	1388013	B1	2/2004
EP	0920627	B1	5/2004
EP	1418003	A1	5/2004
EP	0739240	B1	6/2004
EP	1462800	A1	9/2004
EP	0919812	B1	10/2004
EP	1561507	A1	8/2005
EP	1409727	B1	11/2005
EP	1272668	B1	2/2007
EP	1754788	A2	2/2007
EP	1757694	A2	2/2007
EP	1409745	B1	4/2007
EP	1754788	A3	4/2007
EP	1770171	A1	4/2007
EP	1313882	B1	5/2007
EP	1803822	A1	7/2007
EP	951645	B1	8/2007
EP	1813681	A2	8/2007
EP	1832661	A1	9/2007
EP	1757694	A3	2/2008
EP	2161347	A3	6/2010
WO	WO 90/06509	A1	6/1990
WO	WO 91/07660	A1	5/1991
WO	WO 91/16452	A1	10/1991
WO	WO 93/22053	A1	11/1993
WO	WO 94/29707	A1	12/1994
WO	WO 95/09245	A1	4/1995
WO	WO 97/46882	A1	12/1997
WO	WO 98/02528	A1	1/1998
WO	WO 98/10267	A1	3/1998
WO	WO 99/22868	A1	5/1999
WO	WO 99/44064	A1	9/1999
WO	WO 99/61888	A2	12/1999
WO	WO 00/40750	A1	7/2000
WO	WO 00/62931	A1	10/2000
WO	WO 01/35071	A2	5/2001
WO	WO 01/51668	A1	7/2001
WO	WO 99/61888	A3	12/2001
WO	WO 01/35071	A3	2/2002
WO	WO 02/12896	A1	2/2002
WO	WO 02/28523	A2	4/2002
WO	WO 02/30562	A1	4/2002
WO	WO 02/31506	A1	4/2002
WO	WO 02/44318	A1	6/2002
WO	WO 02/073204	A2	9/2002
WO	WO 03/018757	A2	3/2003
WO	WO 03/019141	A2	3/2003
WO	WO 03/020974	A2	3/2003
WO	WO 03/020986	A1	3/2003
WO	WO 03/023057	A2	3/2003
WO	WO 03/031938	A2	4/2003
WO	WO 03/035894	A2	5/2003
WO	WO 03/035895	A2	5/2003
WO	WO 03/044217	A2	5/2003
WO	WO 03/044224	A1	5/2003
WO	03048295	A1	6/2003
WO	WO 03/069421	A2	8/2003
WO	WO 03/018757	A3	9/2003
WO	WO 02/073204	A3	10/2003
WO	WO 03/044217	A3	10/2003
WO	WO 03/031938	A3	11/2003
WO	WO 03/093795	A2	11/2003
WO	WO 03/023057	A3	12/2003
WO	WO 03/069421	A3	12/2003
WO	WO 03/035895	A3	1/2004
WO	WO 03/035894	A3	3/2004
WO	WO 2004/025251	A2	3/2004
WO	WO 03/019141	A3	4/2004
WO	WO 2004/029221	A2	4/2004
WO	WO 2004/029221	A3	5/2004
WO	WO 2004/037374	A2	5/2004
WO	WO 2004/044236	A1	5/2004
WO	WO 2004/056978	A1	7/2004
WO	2004065629	A1	8/2004
WO	WO 2004/076643	A2	9/2004
WO	WO 03/093795	A3	10/2004
WO	WO 2004/037374	A3	10/2004
WO	WO 2004/088310	A1	10/2004
WO	WO 2004/025251	A3	11/2004
WO	WO 2004/101762	A2	11/2004
WO	WO 2004/113877	A1	12/2004
WO	WO 2004/101762	A3	2/2005
WO	WO 2005/028663	A2	3/2005
WO	WO 2005/042713	A2	5/2005
WO	WO 2005/043121	A2	5/2005
WO	WO 2005/047529	A1	5/2005
WO	WO 2005/047532	A1	5/2005
WO	WO 2005/023091	A3	6/2005
WO	WO 2005/049168	A2	6/2005
WO	WO 2005/058937	A2	6/2005
WO	WO 2005/061075	A1	7/2005
WO	WO 2005/049168	A3	9/2005
WO	WO 2005/084374	A2	9/2005

WO	WO 2005/084380	A2	9/2005
WO	WO 2005/085476	A1	9/2005
WO	WO 2005/085861	A2	9/2005
WO	WO 2005/098046	A2	10/2005
WO	WO 2005/108621	A1	11/2005
WO	WO 2005/109238	A2	11/2005
WO	WO 2005/028663	A3	12/2005
WO	WO 2005/098046	A3	12/2005
WO	WO 2005/116264	A2	12/2005
WO	WO 2005/118852	A2	12/2005
WO	WO 2005/121362	A2	12/2005
WO	WO 2005/085861	A3	2/2006
WO	WO 2006/010610	A2	2/2006
WO	WO 2006/023563	A2	3/2006
WO	WO 2005/121362	A3	4/2006
WO	WO 2006/041453	A1	4/2006
WO	WO 2006/043181	A2	4/2006
WO	WO 2005/109238	A3	6/2006
WO	WO 2006/010610	A3	6/2006
WO	WO 2006/043181	A3	6/2006
WO	WO 2006/076567	A2	7/2006
WO	WO 2006/078470	A2	7/2006
WO	WO 2005/043121	A3	8/2006
WO	2006097049	A1	9/2006
WO	WO 2006/076567	A3	9/2006
WO	WO 2006/078470	A3	9/2006
WO	WO 2006/100366	A2	9/2006
WO	WO 2005/042713	A3	11/2006
WO	WO 2006/023563	A3	11/2006
WO	WO 2006/120434	A1	11/2006
WO	WO 2005/084380	A3	12/2006
WO	WO 2005/116264	A3	2/2007
WO	WO 2007/020081	A1	2/2007
WO	WO 2004/076643	A3	3/2007
WO	WO 2007/024264	A2	3/2007
WO	WO 2007/028146	A2	3/2007
WO	WO 2007/030949	A2	3/2007
WO	WO 2007/033167	A2	3/2007
WO	WO 2007/034221	A2	3/2007
WO	WO 2007/035414	A2	3/2007
WO	WO 2007/024264	A3	4/2007
WO	WO 2007/036025	A1	4/2007
WO	WO 2007/038264	A2	4/2007
WO	WO 2007/041610	A2	4/2007
WO	WO 2007/044690	A2	4/2007
WO	WO 2007/048076	A2	4/2007
WO	WO 2007/030949	A3	5/2007
WO	WO 2007/034221	A3	5/2007
WO	WO 2007/050495	A2	5/2007
WO	WO 2007/053142	A1	5/2007
WO	WO 2007/053648	A2	5/2007
WO	WO 2007/053785	A2	5/2007
WO	WO 2007/059430	A2	5/2007
WO	WO 2007/062222	A2	5/2007
WO	WO 2005/058937	A3	6/2007
WO	WO 2007/067734	A2	6/2007
WO	WO 2007/048076	A3	7/2007
WO	WO 2007/053648	A3	7/2007
WO	WO 2007/075879	A2	7/2007
WO	WO 2007/076989	A1	7/2007
WO	WO 2007/079229	A2	7/2007
WO	WO 2007/079250	A2	7/2007
WO	WO 2007/080583	A2	7/2007
WO	WO 2007/082144	A2	7/2007
WO	WO 2007/082154	A2	7/2007
WO	WO 2007/082379	A2	7/2007
WO	WO 2007/050495	A3	8/2007
WO	WO 2007/075879	A3	8/2007
WO	WO 2007/087612	A2	8/2007
WO	WO 2007/089880	A2	8/2007
WO	WO 2007/089911	A2	8/2007
WO	WO 2007/090670	A1	8/2007
WO	WO 2007/092713	A2	8/2007
WO	WO 2007/098484	A2	8/2007
WO	WO 2006/100366	A3	9/2007
WO	WO 2007/100684	A2	9/2007
WO	WO 2007/101609	A1	9/2007
WO	WO 2007/033167	A3	10/2007
WO	WO 2007/038264	A3	10/2007
WO	WO 2007/044690	A3	10/2007
WO	WO 2007/053785	A3	10/2007
WO	WO 2007/059430	A3	10/2007
WO	WO 2005/084374	A3	11/2007
WO	WO 2007/035414	A3	11/2007
WO	WO 2007/044091	A3	11/2007
WO	WO 2007/089880	A3	11/2007
WO	WO 2007/126938	A2	11/2007
WO	WO 2007/132166	A2	11/2007
WO	WO 2007/132167	A2	11/2007
WO	WO 2007/082379	A3	12/2007
WO	WO 2007/098484	A3	12/2007
WO	WO 2007/062222	A3	1/2008
WO	WO 2007/100684	A3	1/2008
WO	WO 2007/075836	A3	2/2008
WO	WO 2008/017871	A1	2/2008
WO	WO 2007/089911	A3	5/2008
WO	WO 2007/028146	A3	6/2008
WO	WO 2007/067734	A3	8/2008
WO	WO 2007/126938	A3	10/2008
WO	WO 2007/082154	A3	11/2008
WO	WO 2007/087612	A3	11/2008
WO	WO 2007/082144	A3	12/2008
WO	WO 2007/092713	A3	12/2008
WO	2009013492	A1	1/2009
WO	2009013496	A1	1/2009
WO	WO 2007/079229	A3	1/2009
WO	WO 2007/080583	A3	2/2009
WO	WO 2007/079250	A3	3/2009
WO	WO 2007/041610	A3	4/2009
WO	WO 2009/019455	A3	4/2009

OTHER PUBLICATIONS

U.S. Appl. No. 11/825,677, filed Jul. 5, 2007, Lopez et al.

U.S. Appl. No. 11/909,959, filed Sep. 27, 2007, Duff.

U.S. Appl. No. 60/764,420, filed Feb. 2, 2005, Quake.

U.S. Appl. No. 60/949,227, filed Jul. 11, 2007, Kapur.

Adinolfi, et al. Gene Amplification to Detect Fetal Nucleated Cells in Pregnant Women. The Lancet. Aug. 5, 1989:328-329.

Adinolfi, et al. Rapid detection of aneuploidies by microsatellite and the quantitative fluorescent polymerase chain reaction. Prenat. Diagn. 1997; 17(13):1299-311.

Adinolfi, M. On a Non-Invasive Approach to Prenatel Diagnosis based on the detection of Fetal Nucleated Cells in Maternal Blood Samples. Prenatal Diagnosis. 1991;11:799-804.

Ahn, et al. A fully integrated micromachined magnetic particle separator. Journal of Microelectromechanical Systems. 1996; 5(3):151-158.

Andrews, et al. Enrichment of fetal nucleated cells from maternal blood: model test system using cord blood. Prenatal Diagnosis. 1995; 15:913-919.

Applicant's Amendment and Response dated Jun. 17, 2009 to Non-Final Office Action of Jan. 28, 2009 re U.S. Appl. No. 11/701,686.

Ariga, et al. Kinetics of fetal cellular and cell-free DNA in the maternal circulation during and after pregnancy: implications for noninvasive prenatal diagnosis. Transfusion. 2001; 41:1524-1530.

Arnould, et al. Agreement between chromogenic in situ hybridisation (CISH) and FISH in the determination of HER2 status in breast cancer. Br J Cancer. 2003; 88(10):1587-91. (Abstract only).

Babochkina, et al. Direct detection of fetal cells in maternal blood: a reappraisal using a combination of two different Y chromosome-specific FISH probes and a single X chromosome-specific probe. Arch Gynecol Obstet. Dec. 2005;273(3):166-9. (Abstract only).

Babochkina, T. I. Ph. D. Dissertation—Fetal cells in maternal circulation: Fetal cell separation and FISH analysis. University of Basel, Switzerland. Dec. 8, 2005. (123 pages).

Balko, et al. Gene expression patterns that predict sensitivity to epidermal growth factor receptor tyrosine kinase inhibitors in lung cancer cell lines and human lung tumors. BMC Genomics. Nov. 10, 2006;7:289 (14 pages).

Barrett, et al. Comparative genomic hybridization using oligonucleotide microarrays and total genomic DNA. Proc Natl Acad Sci U S A. 2004; 101(51):17765-70.

Basch, et al. Cell separation using positive immunoselective techniques. Journal of Immunological Methods. 1983;56:269-280.

Bauer, J. Advances in cell separation: recent developments in counterflow centrifugal elutriation and continuous flow cell separation. Journal of Chromatography. 1999;722:55-69.
Becker, et al. Fabrication of Microstructures With High Aspect Ratios and Great Structural Heights by Synchrotron Radiation Lithography, Galvanoforming, and Plastic Moulding (LIGA Process). Microelectronic Engineering. 1986;4:35-56.
Becker, et al. Planar quartz chips with submicron channels for two-dimensional capillary electrophoresis applications. J. Micromech Microeng.1998;9:24-28.
Beebe et al. Functional Hydrogel Structures for Autonomous Flow Control Inside Microfluidic Channels. Nature. 2000; 404:588-590.
Bennett, et al. Toward the 1,000 dollars human genome. Pharmacogenomics. 2005; 6(4):373-82.
Berenson, et al. Cellular Immunoabsorption Using Monoclonal Antibodies. Transplantation.1984;38:136-143.
Berenson, et al. Positive selection of viable cell populations using avidin-biotin immunoadsorption. Journal of Immunological Methods. 1986;91:11-19.
Berg, H. C. Random Walks in Biology, Ch. 4. Princeton University Press. Princeton, NJ. 1993. pp. 48-64.
Berger, et al. Design of a microfabricated magnetic cell separator. Electrophoresis. Oct. 2001;22(18):3883-92.
Bianchi, et al. Isolation of fetal DNA from nucleated erythrocytes in maternal blood. Medical Sciences. 1990;87:3279-3283.
Bianchi, et al. Demonstration of fetal gene sequences in nucleated erythrocytes isolated from maternal blood. American Journal of Human Genetics. 1989;45:A252.
Bianchi, et al. Fetal gender and aneuploidy detection using fetal cells in maternal blood: analysis of NIFTY I data Prenatal Diagnosis. 2002; 22:609-615.
Bianchi, et al. Fetal nucleated erythrocytes (FNRBC) in maternal blood: erythroid-specific antibodies improve detection. The American Journal of Human Genetics. Oct. 1992. Supplemental to vol. 51, No. 4: 996.
Bianchi, et al. Isolation of Male Fetal DNA from Nucleated Erythrocytes (NRBC) in Maternal Blood. The American Pediatric Society and Society for Pediatric Research, Mar. 1989; 818:139A.
Bianchi, et al. Possible Effects of Gestational Age on the Detection of Fetal Nucleated Erythrocytes in Maternal Blood. Prenatal Diagnosis. 1991;11:523-528.
Bignell, et al. High-resolution analysis of DNA copy number using oligonucleotide microarrays. Genome Research. 2004; 14(2):287-295.
Blake, et al. Assessment of multiplex fluorescent PCR for screening single cells for trisomy 21 and single gene defects. Mol. Hum. Reprod. 1999; 5(12):1166-75.
Bode, et al. Mutations in the tyrosine kinase domain of the EGFR gene are rare in synovial sarcoma. Mod Pathol. Apr. 2006;19(4):541-7.
Boehm, et al. Analysis of Defective *Dystrophin* Genes with cDNA Probes: Rearrangement Polymorphism, Detection of Deletions in Carrier Females, and Lower Than Expected Frequency of Carrier Mothers in Isolated Cases of Delections. Pediatric Research. Apr. 1989: 139A-820.
Bohmer, et al. Differential Development of Fetal and Adult Haemoglobin Profiles in Colony Culture: Isolation of Fetal Nucleated Red Cells by Two-Colour Fluorescence Labelling. Br. J. Haematol. 1998; 103:351-60.
Brison, et al. General Method for Cloning Amplified DNA by Differential Screening with Genomic Probes. Molecular and Cellular Biology. 1982;2:578-587.
Brody, et al. Deformation and Flow of Red Blood Cells in a Synthetic Lattice: Evidence for an Active Cytoskeleton. Biophys. J. 68:2224-2232 (1995).
Caggana, M. Microfabricated devices for sparse cell isolation. CNF Project #905-00. Cornell NanoScale Facility. 2003; pp. 38-39.
Caggana, M. Microfabricated devices for sparse cell isolation. CNF Project #905-00. Cornell NanoScale Facility. 2004-2005; pp. 32-33.
Calin, et al. A microRNA signature Associated with prognosis and progression in chronic lymphocytic leukemia. New England Journal of Medicine. 2005; 353:1793-1801.
Cha, The utility of an erythroblast scoring system and gender-independent short tandem repeat (STR) analysis for the detection of aneuploid fetal cells in maternal blood. Prenat. Diagn. 2005; 25(7):586-91.
Chamberlain, et al. Deletion screening of the Duchenne muscular dystrophy locus via multiplex DNA amplification. Nucleic Acids Research. 1988;16:11141-11156.
Chan, et al. Size distributions of maternal and fetal DNA in maternal plasma. Clin Chem. Jan. 2004;50(1):88-92.
Chang, et al. Biomimetic technique for adhesion-based collection and separation of cells in a microfluidic channel. Lab Chip. 2005; 5:64-73.
Cheung, et al. Development and validation of a CGH microarray for clinical cytogenetic diagnosis. Genet Med. 2005; 7(6):422-32.
Chiu, et al. Patterned Deposition of Cells and Proteins Onto Surfaces by Using Three-Dimensional Microfluidic Systems. Proceedings of the National Academy of Sciences of the United States of America. 2000; pp. 2408-2413.
Choesmel, et al. Enrichment methods to detect bone marrow micrometastases in breast carcinoma patients: clinical relevance. Breast Cancer Res. 2004;6(5):R556-569.
Choolani, et al. Characterization of First Trimester Fetal Erythroblasts for Non-Invasive Prenatal Diagnosis. Mol. Hum. Reprod. 2003; 9:227-35.
Chou, et al. A Microfabricated Device for Sizing and Sorting DNA Molecules. Proceedings of the National Academy of Sciences of the United States of America. 1999; pp. 11-13.
Chou, et al. Sorting by diffusion: An asymmetric obstacle course for continuous molecular separation. PNAS. 1999; 96(24):13762-13765.
Christel, et al. High aspect ratio silicon microstructures for nucleic acid extraction. Solid-state sensor and actuator workshop. Hilton Head, SC. Jun. 8-11, 1998; 363-366.
Christensen, et al. Fetal Cells in Maternal Blood: A Comparison of Methods for Cell Isolation and Identification. Fetal Diagn. Ther. 2005; 20:106-12.
Chueh, et al. Prenatal Diagnosis Using Fetal Cells from the Maternal Circulation. West J. Med. 159:308-311 (1993).
Chueh, et al. Prenatal Diagnosis Using Fetal Cells in the Maternal Circulation. Seminars in Perinatology. 1990;14:471-482.
Chueh, et al. The search for fetal cells in the maternal circulation. J Perinat Med. 1991;19:411-420.
Cirigliano, et al. "Clinical application of multiplex quantitative fluorescent polymerase chain reaction (QF-PCR) for the rapid prenatal detection of common chromosome aneuploidies," Molecular Human Reproduction, 2001, vol. 7, No. 10, 1001-1006.
Clayton, et al. Fetal Erythrocytes in the Maternal Circulation of Pregnant Women. Obstetrics and Gynecology. 1964;23:915-919.
Collarini, et al. Comparison of methods for erythroblast selection: application to selecting fetal erythroblasts from maternal blood. Cytometry. 2001; 45:267-276.
Cremer, et al. Detection of chromosome aberrations in human interphase nucleus by visualization of specific target DNAs with radioactive and non-radioactive in situ hybridization techniques: diagnosis of trisomy 18 with probe L1.84. Human Genetics. 1986;74:346-352.
Cremer, et al. Detection of chromosome aberaations in metaphase and interphase tumor cells by in situ hybridization using chromosome-specific library probes. Human Genetics.1988;80:235-246.
Das, et al. Dielectrophoretic segregation of different human cell types on microscope slides. Anal. Chem. 2005; 77:2708-2719.
De Alba, et al. Prenatal diagnosis on fetal cells obtained from maternal peripheral blood: report of 66 cases. Prenat Diagn. Oct. 1999;19(10):934-40.
De Kretser, et al. The Separation of Cell Populations using Monoclonal Antibodies attached to Sepharose. Tissue Antigens. 1980;16:317-325.
Delamarche, et al. Microfluidic Networks for Chemical Patterning of Substrates: Design and Application to Bioassays. Journal of the American Chemical Society. 1998; 120:500-508.
Delamarche, et al. Patterned Delivery of Immunoglobulins to Surfaces Using Microfluidic Networks. Science. 1997; 276:779-781.

Deng, et al. Enumeration and microfluidic chip separation of circulating fetal cells early in pregnancy from maternal blood. American Journal of Obstetrics & Gynecology. Dec. 2008 (vol. 199, Issue 6, p. S134).
Deshmukh, et al. Continuous Micromixer With Pulsatile Micropumps. Solid-State Sensor and Actuator Workshop. Hilton Head Island, South Carolina; Jun. 4-8, 2000:73-76.
Di Naro, et al. Prenatal diagnosis of beta-thalassaemia using fetal erythroblasts enriched from maternal blood by a novel gradient. Mol Hum Reprod. 2000; 6(6):571-4.
Dilella, et al. Screening for Phenylketonuria Mutations by DNA Amplification with the Polymerase Chain Reaction. The Lancet. Mar. 5, 1988:497-499.
Doyle, et al. Self-Assembled Magnetic Matrices for DNA Separation Chips. Science 295:2237 (2002).
Eigen, et al. Sorting Single Molecules: Application to Diagnostics and Evolutionary Biotechnology. Proceedings of the National Academy of Sciences of the United States of America. 1994; 91:5740-5747.
Emanuel, et al. Amplification of Specific Gene Products from Human Serum. GATA, 1993, vol. 10, No. 6, 144-146.
European search report dated Nov. 9, 2009 for Application No. 7784442.1.
European search report dated Dec. 21, 2009 for Application No. 07798579.4.
European search report dated Dec. 22, 2009 for Appllication No. 07798580.2.
European search report dated Dec. 22, 2009 for Application No. 0778444.7.
European Search Report dated Jul. 31, 2009 for EP07763674.4.
Final Office Action dated Sep. 11, 2009 issued in U.S. Appl. No. 11/701,686.
Non-Final Office Action dated Jan. 28, 2009 issued in U.S. Appl. No. 11/701,686.
Falcidia, et al. Fetal Cells in maternal blood: a six-fold increase in women who have undergone mniocentesis and carry a fetus with Down syndrome: a multicenter study. Neuropediatrics. 2004; 35(6):321-324. (Abstract only).
Fan, et al. Highly parallel SNP genotyping. Cold Spring Harb. Symp. Quant. Biol. 2003; 68:69-78.
Fan, et al. Single cell degenerate oligonucleotide primer-PCR and comparative genomic hybridization with modified control reference. Journal of Ahejian University—Science A. 2001; 2(3):318-321.
Farber, et al. Demonstration of spontaneous XX/XY chimerism by DNA fingerprinting. Human Genetics. 1989;82:197-198.
Farooqui, et al. Microfabrication of Submicron Nozzles in Silicon Nitride. Journal of Microelectromechanical Systems. 1992; 1(2):86-88.
Fiedler, et al. Dielectrophoretic Sorting of Particles and Cells in a Microsystem. Analytical Chemistry. 1998; pp. 1909-1915.
Findlay, et al. Using MF-PCR to diagnose multiple defects from single cells: implications for PGD. Mol Cell Endocrinol. 2001; 183 Suppl 1:S5-12.
Freemantle, M. Downsizing Chemistry. Chemical analysis and synthesis on microchips promise a variety of potential benefits. Chemical & Engineering News. 1999; pp. 27-36.
Fu, et al. An integrated miscrofabricated cell sorter. Anal Chem. 2002;74:2451-2457.
Fu, et al. A Microfabricated Fluorescence-Activated Cell Sorter. Nature Biotechnology.1999; 17:1109-1111.
Fuhr, et al. Biological Application of Microstructures. Topics in Current Chemistry. 1997; 194:83-116.
Furdui, et al. Immunomagnetic T cell capture from blood for PCR analysis using microfluidic systems. Lab Chip. Dec. 2004;4(6):614-8.
Ganshirt-Ahlert, et al. Magnetic cell sorting and the transferrin receptor as potential means of prenatal diagnosis from maternal blood. Am J Obstet Gynecol. 1992;166:1350-1355.
Ganshirt-Ahlert, et al. Noninvasive prenatal diagnosis: Triple density gradient, magnetic activated cell sorting and FISH prove to be an efficient and reproducible method for detection of fetal aneuploidies from maternal blood. The American Journal of Human Genetics. Oct. 1992. Supplemental to vol. 51, No. 4: 182.
GenomeWeb. Immunicon inks biomarker assay, lab services deal with merck serona. Available at C:\Documents and Settings\fc3\Local Settings\Temporary InternetFiles\OLK35E\141896-1.htm. Accessed on Sep. 11, 2007.
Ghia, et al. Ordering of human bone marrow B lymphocyte precursors by single-cell polymerase chain reaction analyses of the rearrangement status of the immunoglobulin H and L chain gene loci. J Exp Med. Dec. 1, 1996;184(6):2217-29.
Giddings, J. C. Chemistry 'Eddy' Diffusion in Chromatography. Nature. 1959;184:357-358.
Giddings, J. C. Field-Flow Fractionation: Analysis of Macromolecular, Colloidal, and Particulate Materials. Science. 1993;260:1456-1465.
Gonzalez, et al. Multiple displacement amplification as a pre-polymerase chain reaction (pre-PCR) to process difficult to amplify samples and low copy number sequences from natural environments. Environ Microbiol. 2005; 7(7):1024-8.
Graham. Efficiency comparison of two preparative mechanisms for magnetic separation of erthrocytes from whole blood. J. Appl. Phys. 1981; 52:2578-2580.
Greaves, et al. Expression of the OKT Monoclonal Antibody Defined Antigenic Determinants in Malignancy. Int. J. Immunopharmac. 1981;3:283-299.
Guetta, et al. Analysis of fetal blood cells in the maternal circulation: challenges, ongoing efforts, and potential solutions. Stem Cells Dev. 2004;13(1):93-9.
Gunderson, et al. A genome-wide scalable SNP genotyping assay using microarray technology. Nat Genet. 2005; 37(5):549-54.
Hahn, et al. Current applications of single-cell PCR. Cell. Mol. Life Sci. 2000; 57(1):96-105. Review.
Hamabe, et al. Molecular study of the Prader-Willi syndrome: deletion, RFLP, and phenotype analyses of 50 patients. Am J Med Genet. Oct. 1, 1991;41(1):54-63.
Han, et al. Separation of Long DNA Molecules in a Microfabricated Entropic Trap Array. Science. 2000;288:1026-1029.
Hardenbol, et al. Highly multiplexed molecular inversion probe genotyping: over 10,000 targeted SNPs genotyped in a single tube assay. Genome Res. 2005;15(2):269-75.
Hardenbol, et al. Multiplexed genotyping with sequence-tagged molecular inversion probes. Nat. Biotechnol. 2003; 21(6):673-8.
Hartmann, et al. Gene expression profiling of single cells on large-scale oligonucleotide arrays. Nucleic Acids Research. 2006; 34(21): e143. (11 pages).
Herzenberg, et al. Fetal cells in the blood of pregnant women: Detection and enrichment by flourescence-activated cell sorting. Proc. Natl. Acad. Sci. 1979;76:1453-1455.
Holzgreve, et al. Fetal Cells in the Maternal Circulation. Journal of Reproductive Medicine. 1992;37:410-418.
Hong, et al. A nanoliter-scale nucleic acid processor with parallel architecture. Nat. Biotechnol. 2004; 22(4):435-9.
Hosono, et al. Unbiased whole-genome amplification directly from clinical samples. Genome Res. May 2003;13(5):954-64.
Hromadnikova, et al. "Quantitative analysis of DNA levels in maternal plasma in normal and Down syndrome pregnancies." Bio Med Central, May 2002, 1-5.
Huang, et al. A DNA prism for high-speed continuous fractionation of large DNA molecules. Nature Biotechnology. 2002;20:1048-1051.
Huang, et al. Continuous Particle Separation Through Deterministic Lateral Displacement. Science 304:987-90 (2004).
Huang, et al. Electric Manipulation of Bioparticles and Macromoledules on Microfabricated Electrodes. Analytical Chemistry. 2001; pp. 1549-1559.
Huang, et al. Role of Molecular Size in Ratchet Fractionation. 2002; 89(17):178301-1-178301-4.
Huh, et al. Gravity-driven microhydrodynamics-based cell sorter (microHYCS) for rapid, inexpensive, and efficient cell separation and size-profiling. 2nd Annual International IEEE-EMBS Special Topic Conference on Microtechnology in Medicine and Biology. Madison, Wisconsin USA; May 2-4, 2002:466-469.
Hviid T. In-Cell PCT method for specific genotyping of genomic DNA from one individual in a micture of cells from two individuals:

a model study with specific relevance to prenatal diagnosis based on fetal cells in maternal blood. Molecular Diagnostics and Genetics. 2002; 48:2115-2123.
Hviid, T. In-cell polymerase chain reaction: strategy and diagnostic applications. Methods Mol Biol. 2006;336:45-58.
International search report and written opinion dated Mar. 16, 2010 for PCT Application No. U52009/57136.
International Search Report and Written Opinion dated Sep. 18, 2008 for PCT/US2007/003209.
International search report dated Jan. 16, 2008 for PCT Application No. US2007/71247.
International search report dated Jan. 25, 2008 for PCT Application No. US2007/71250.
International search report dated Nov. 15, 2007 for PCT Application No. US2007/71149.
International search report dated Nov. 26, 2007 for PCT Application No. US2007/71256.
International search report dated Feb. 25, 2008 for PCT Application No. US07/71148.
International search report dated Feb. 25, 2008 for PCT Application No. US2007/71248.
Iverson, et al. Detection and Isolation of Fetal Cells From Maternal Blood Using the Flourescence-Activated Cell Sorter (FACS). Prenatal Diagnosis 1981;1:61-73.
Jan, et al. Fetal Erythrocytes Detected and Separated from Maternal Blood by Electronic Fluorescent Cell Sorter. Texas Rep Biol Med. 1973;31:575.
Jeon, et al. Generation of Solution and surface Gradients Using Microfluidic Systems. Langmuir. 2000, pp. 8311-8316.
Jiang, et al. Genome amplification of single sperm using multiple displacement amplification. Nucleic Acids Res. 2005; 33(10):e91. (9 pages).
Kamholz, et al. Quantitative Analysis of Molecular Interaction in a Microfluidic Channel: the T-Sensor. Analytical Chemistry. 1999; pp. 5340-5347.
Kan, et al. Concentration of Fetal Red Blood Cells From a Mixture of Maternal and Fetal Blood by Anti-i Serum—An Aid to Prenatal Diagnosis of Hemoglobinopathies. Blood. 1974; 43:411-415.
Kasakov, et al. Extracellular DNA in the blood of pregnant women. Tsitologiia. 1995;37(3):232-6. (English translation only).
Kenis, et al. Microfabrication Inside Capillaries Using Multiphase Laminar Flow Patterning. Science. 1999; 285:83-85.
Kim, et al. Polymer microstructures formed by moulding in capillaries. Nature. 1995;376:581-584.
Kimura, et al. The DYRK1A gene, encoded in chromosome 21 Down syndrome critical region, bridges between (β-amyloid production and tau phosphorylation in Alzheimer disease. Human Molecular Genetics, Nov. 29, 2008, vol. 16, No. 1, 15-23.
Klein, C. A. Single cell amplification methods for the study of cancer and cellular ageing. Mech. Ageing Dev. 2005; 126(1):147-51.
Klein, et al. Comparative genomic hybridization, loss of heterozygosity, and DNA sequence analysis of single cells. Proc Natl Acad Sci U S A. 1999; 96(8):4494-9.
Kogan, et al. An Improved Method for Prenatal Diagnosis of Genetic Diseases by Analysis of Amplified DNA Sequences, Application to Hemophilia A. The New England Journal of Medicine. 1987;317:985-990.
Korenberg, et al. Down syndrome phenotypes: the consequences of chromosomal imbalance. PNAS 1994; 91:4997-5001.
Krabchi, et al. Quantification of all fetal nucleated cells in maternal blood between the 18th and 22nd weeks of pregnancy using molecular cytogenic techniques. Clin. Genet. 2001; 60:145-150.
Krivacic, et al. A rare-cell detector for cancer. PNAS. 2004;101:10501-10504.
Kulozik, et al. Fetal Cell in the Maternal Circulation: Detection by Direct AFP-Immunoflourescence. Human Genetics. 1982;62:221-224.
Leutwyler, K. Mapping Chromosome 21. Available at http://www.scientificamerican.com/article.cfm?id=mapping-chromosome-21. Accessed Feb. 3, 2010.
Levett, et al. A large-scale evaluation of amnio-PCR for the rapid prenatal diagnosis of fetal trisomy. Ultrasound Obstet Gynecol. 2001; 17(2):115-8.
Li ,et al. Transport, Manipulation, and Reaction of Biological Cells On-Chip Using Electrokinetic Effects. Analytical Chemistry., 1997; pp. 1564-1568.
Li, et al. Amplification and analysis of DNA sequences in single human sperm and diploid cells. Nature. 1988;335:414-417.
Lichter , et al. Delineation of individual human chromosomes in metaphase and interphase cells by in situ suppression hyridization using recombinant DNA libraries. Hum Genet. 1988;80:224-234.
Liu, et al. Development and validation of a T7 based linear amplification for genomic DNA. BMC Genomics. 2003; 4(1):19. (11 pages).
Loken , et al. Flow Cytometric Analysis of Human Bone Marrow: I. Normal Erythroid Development. Blood. 1987;69:255-263.
Mahr, et al. Fluorescence in situ hybridization of fetal nucleated red blood cells. The American Journal of Human Genetics. Oct. 1992. Supplemental to vol. 51, No. 4: 1621.
Maloney et al. "Microchimerism of maternal origin persists into adult life," J. Clin. Invest. 104:41-47 (1999).
Margulies, et al. Genome sequencing in microfabricated high-density picolitre reactors. Nature. 2005; 437:376-80.
Martin, et al. "A method for using serum or plasma as a source of NDA for HLA typing," Human Immunology. 1992; 33:108-113.
Mavrou, et al. Identification of nucleated red blood cells in maternal circulation: A second step in screening for fetal aneuploidies and pregnancy complications. Prenat Diagn. 2007; 247:150-153.
McCabe, et al. DNA microextraction from dried blood spots on filter paper blotters: potential applications to newborn screening. Hum Genet.1987;75:213-216.
McCarley, et al. Patterning of surface-capture architectures in polymer-based microanalytical devices. In Kuther, et al. Eds. Royal Society of Chemistry Special Publication. 2005;130-132. (Abstract only).
Mehrishi , et al. Electrophoresis of cells and the biological relevance of surface charge. Electrophoresis.2002;23:1984-1994.
Melville, et al. Direct magnetic separation of red cells from whole blood. Nature. 1975; 255:706.
Mohamed, et al. A Micromachined Sparse Cell Isolation Device: Application in Prenatal Diagnostics. Nanotech 2006 vol. 2; 641-644. (Abstract only).
Mohamed, et al. Biochip for separating fetal cells from maternal circulation. J Chromatogr A. Aug. 31, 2007;1162(2):187-92.
Mohamed, et al. Development of a rare cell fractionation device: application for cancer detection. IEEE Trans Nanobioscience. 2004; 3(4): 251-6.
Moore, et al. Lymphocyte fractionation using immunomagnetic colloid and a dipole magnet flow cell sorter. J Biochem Biophys Methods. 1998;37:11-33.
Moorhead, et al. Optimal genotype determination in highly multiplexed SNP data Eur. J. Hum. Genet. 2006;14(2):207-15. (published online Nov. 23, 2005).
Mueller , et al. Isolation of fetal trophoblast cells from peripheral blood of pregnant women. The Lancet. 1990;336:197-200.
Muller, et al. Moderately repeated DNA sequences specific for the short arm of the human Y chromosome are present in XX makes and reduced in copy number in an XY female. 1986;14:1325-1340.
Mullis, et al. Specific Enzymatic Amplification of DNA In Vitro: The Polymerase Chain Reaction. Cold Spring Harbor Symposia on Quantitative Biolgy 1986;51:263-273.
Murakami, et al. A novel single cell PCR assay: detection of human T lymphotropic virus type I DNA in lymphocytes of patients with adult T cell leukemia. Leukemia. Oct. 1998;12(10):1645-50.
Murthy, et al. Assessment of multiple displacement amplification for polymorphism discovery and haplotype determination at a highly polymorphic locus, MC1R. Hum. Mutat. 2005; 26(2):145-52.
Nagrath, et al. Isolation of rare circulating tumour cells in cancer patients by microchip technology. Nature. 2007; 450: 1235-1241 (with Supplemental pp. 1-10).
Nelson, et al. Genotyping Fetal DNA by Non-Invasive Means: Extraction From Maternal Plasma. Vox Sang. 2001;80:112-116.
Newcombe, R. G. Two-sided confidence intervals for the single proportion: comparison of seven methods. Statistics in Medicine. 1998; 17:857-872.
Oakey et al. Laminar Flow-Based Separations at the Microscale. Biotechnology Progress. 2002; pp. 1439-1442.
Office action dated Nov. 3, 2009 for U.S. Appl. No. 11/763,133.

Office action dated Dec. 1, 2009 for U.S. Appl. No. 11/763,426.
Office action dated Mar. 11, 2010 for U.S. Appl. No. 11/763,245.
Office action dated Mar. 4, 2009 for U.S. Appl. No. 11/228,454.
Office action dated May 4, 2009 for U.S. Appl. No. 11/763,431.
Olson, et al. An In Situ Flow Cytometer for the Optical Analysis of Individual Particles in Seawater. Available at http://www.whoi.edu/science/B/Olsonlab/insitu2001.htm. Accessed Apr. 24, 2006.
Oosterwijk, et al. Prenatal diagnosis of trisomy 13 on fetal cells obtained from maternal blood after minor enrichment. Prenat Diagn. 1998;18(10):1082-5.
Owen, et al. High gradient magnetic separation of erythrocytes. Biophys. J. 1978; 22:171-178.
Pallavicini, et al. Analysis of fetal cells sorted from maternal blood using fluorescence in situ hybridization. The American Journal of Human Genetics. Oct. 1992. Supplemental to vol. 51, No. 4: 1031.
Parano, et al. Fetal Nucleated red blood cell counts in peripheral blood of mothers bearing Down Syndrome fetus. Neuropediatrics. 2001; 32(3):147-149. (Abstract only).
Parano, et al. Noninvasive Prenatal Diagnosis of Chromosomal Aneuploidies by Isolation and Analysis of Fetal Cells from Maternal Blood. Am. J. Med. Genet. 101:262-7 (2001).
Paterlini-Brechot, et al. Circulating tumor cells (CTC) detection: Clinical impact and future directions. Cancer Letter. 2007. (In press, 25 pages.) Available at www.sciencedirect.com.
Paul, et al. Single-molecule dilution and multiple displacement amplification for molecular haplotyping. Biotechniques. 2005; 38(4):553-4, 556, 558-9.
Pawlik, et al. Prodrug Bioactivation and Oncolysis of Diffuse Liver Metastases by a Herpes Simplex Virus 1 Mutant that Expresses the CYP2B1 Transgene. Cancer. 2002;95:1171-81.
Peixoto, et al. Quantification of multiple gene expression in individual cells. Genome Res. Oct. 2004;14(10A):1938-47.
Peng, et al. Real-time detection of gene expression in cancer cells using molecular beacon imaging: new strategies for cancer research. Cancer Res. 2005; 65(5):1909-17.
Petersen, et al. The Promise of Miniaturized Clinical Diagnostic Systems. IVD Technol. 4:43-49 (1998).
Pfaffl, et al. Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res. May 1, 2002;30(9):e36.
Pinkel, et al. Cytogenetic Analysis Using Quantitative, High-sensitivity, Fluorescence Hybridization. Genetics. 1986;83:2934-2938.
Pinkel, et al. Fluorescence in situ Hybridization with Human Chromosome-specific Libraries: Detection of Trisomy 21 and Translocations of Chromosome 4. Genetics.1988;85:9138-9142.
Pinkel, et al. Detection of structural chromosome abberations in metaphase in metaphase spreads and interphase nuclei by in situ hybridization high complexity probes which stain entire human chromosomes. The American Journal of Human Genetics. Sep. 1988. Supplemental to vol. 43, No. 3: 0471.
Pinzani, et al. Isolation by size of epithelial tumor cells in peripheral blood of patients with breast cancer: correlation with real-time reverse transcriptase-polymerase chain reaction results and feasibility of molecular analysis by laser microdissection. Hum Pathol. 2006; 37(6):711-8.
Pohl et al. Principle and applications of digital PCR. Expert Rev Mol Diagn. Jan. 2004;4(1):41-7.
Potti, et al. Genomic signatures to guide the use of chemotherapeutics. Nat Med. 2006; 12(11):1294-1300.
Price, et al. Prenatal Diagnosis with Fetal Cells Isolated from Maternal Blood by Multiparameter Flow Cytometry. Am. J. Obstet. Gynecol. 1991; 165:1731-7.
Prieto, et al. Isolation of fetal nucleated red blood cells from maternal blood in normal and aneuploid pregnancies. Clin Chem Lab Med. Jul. 2002;40(7):667-72.
Product literature for GEM, a system for blood testing: GEM Premier 3000. Avaiable at http://www.ilus.com/premier_gem3000_iqm.asp. Accessed Apr. 24, 2006.
Purwosunu, et al. Clinical potential for noninvasive prenatal diagnosis through detection of fetal cells in maternal blood. Taiwan J Obstet Gynecol. Mar. 2006;45(1):10-20.
Raeburn, P. Fetal Cells Isolated in Women's Blood. Associated Press (Jul. 28, 1989) [electronic version].
Rahil, et al. Rapid detection of common autosomal aneuploidies by quantitative fluorescent PCR on uncultured amniocytes, European Journal of Human Genetics, 2002, vol. 10, 462-466.
Rickman, et al. Prenatal diagnosis by array-CGH. European Journal of Medical Genetics. 2005; 48:232-240.
Rolle, et al. Increase in number of circulating disseminated epithelia cells after surgery for non-small cell lung cancer monitored by MAINTRAC is a predictor for relapse: a preliminary report. World Journal of Surgical Oncology. 2005; 9 pages.
Ruan, et al. Identification of clinically significant tumor antigens by selecting phage antibody library on tumor cells in situ using laser capture microdissection. Molecular & Cellular Proteomics. 2006; 5(12): 2364-73.
Sakhnini, et al. Magnetic behavior of human erythrocytes at different hemoglobin states. Eur Biophys J. Oct. 2001;30(6):467-70.
Samura, et al. Diagnosis of trisomy 21 in fetal nucleated erythrocytes from maternal blood by use of short tandem repeat sequences. Clin. Chem. 2001; 47(9):1622-6.
Samura, et al. Female fetal cells in maternal blood: use of DNA polymorphisms to prove origin. Hum. Genet. 2000;107(1):28-32.
Sato, et al. Individual and Mass Operation of Biological Cells Using Micromechanical Silicon Devices. Sensors and Actuators. 1990;A21-A23:948-953.
Schaefer, et al. The Clinical Relevance of Nucleated Red Blood Cells counts. Sysmex Journal International. 2000; 10(2):59-63.
Schröder, et al. Fetal Lymphocytes in the Maternal Blood. The Journal of Hematolog:Blood. 1972;39:153-162.
Sethu, et al. Continuous Flow Microfluidic Device for Rapid Erythrocyte Lysis. Anal. Chem. 76:6247-6253 (2004).
Shen, et al. High-throughput SNP genotyping on universal bead arrays. Mutat. Res. 2005; 573:70-82.
Shendure, et al. Accurate multiplex polony sequencing of an evolved bacterial genome. Science. 2005; 309:1728-32.
Sherlock, et al. Assessment of diagnostic quantitative fluorescent multiplex polymerase chain reaction assays performed on single cells. Ann. Hum. Genet. 1998; 62:9-23.
Sitar, et al. The Use of Non-Physiological Conditions to Isolate Fetal Cells from Maternal Blood. Exp. Cell. Res. 2005; 302:153-61.
Sohda, et al. The Proportion of Fetal Nucleated Red Blood Cells in Maternal Blood: Estimation by FACS Analysis. Prenat. Diagn. 1997; 17:743-52.
Stipp, D. IG Labs Licenses New Technology for Fetal Testing. The Wall Street Journal. Aug. 10, 1990:B5.
Stoecklein, et al. SCOMP is superior to degenerated oligonucleotide primed-polymerase chain reaction for global amplification of minute amounts of DNA from microdissected archival tissue samples. Am J Pathol. 2002; 161(1):43-51.
Stoughton, et al. Data-adaptive algorithms for calling alleles in repeat polymorphisms. Electrophoresis. 1997;18(1):1-5.
Sun, et al. Whole-genome amplification: relative efficiencies of the current methods. Leg Med. 2005; 7(5):279-86.
Sykes, et al. Quantitation of targets for PCR by use of limiting dilution. Biotechniques. Sep. 1992;13(3):444-9.
Tettelin, et al. The nucleotide sequence of *Saccharomyces cerevisiae* chromosome VII. Nature. May 29, 1997;387(6632 Suppl):81-4.
Thomas, et al. Specific Binding and Release of Cells from Beads Using Cleavable Tettrametric Antibody Complexes. Journal of Immunological Methods 1989;120:221-231.
Tibbe, et al. Statistical considerations for enumeration of circulating tumor cells. Cytometry A. Mar. 2007;71(3):154-62.
Toner, et al. Blood-on-a-Chip. Annu. Rev. Biomed. Eng. 7:77-103, C1-C3 (2005).
Trask, et al. Detection of DNA Sequences and Nuclei in Suspension by In Situ Hybridization and Dual Beam Flow Cytometry. Science. 1985;230:1401-1403.
Troeger, et al. Approximately half of the erythroblasts in maternal blood are of fetal origin. Mol Hum Reprod. 1999; 5(12):1162-5.
Van Raamsdonk, et al. Optimizing the detection of nascent transcripts by RNA fluorescence in situ hybridization. Nucl. Acids. Res. 2001; 29(8):e42.
Vogelstein, et al. "Digital PCR." Proc Natl. Acad Sci. USA, Aug. 1999, vol. 96., 9236-9241.

Volkmuth, et al. DNA electrophoresis in microlithographic arrays. Nature. 1992; 358:600-602.
Volkmuth, et al. Observation of Electrophoresis of Single DNA Molecules in Nanofabricated Arrays. Presentation at joint annual meeting of Biophysical Society and the American Society for Biochemistry and Molecular Biology. Feb. 9-13, 1992.
Von Eggeling, et al. Determination of the origin of single nucleated cells in maternal circulation by means of random PCR and a set of length polymorphisms. Hum Genet. Feb. 1997;99(2):266-70.
Vona, et al. Enrichment, immunomorphological, and genetic characterization of fetal cells circulating in maternal blood. Am J Pathol. Jan. 2002;160(1):51-8.
Voullaire, et al. Detection of aneuploidy in single cells using comparative genomic hybridization. Prenat Diagn. 1999; 19(9):846-51.
Vrettou, et al. Real-time PCR for single-cell genotyping in sickle cell and thalassemia syndromes as a rapid, accurate, reliable, and widely applicable protocol for preimplantation genetic diagnosis. Human Mutation. 2004; 23(5):513-21.
Wachtel, et al. Fetal Cells in the Maternal Circulation: Isolation by Multiparameter Flow Cytometry and Confirmation by Polymerase Chain Reaction. Human Reproduction. 1991;6:1466-1469.
Wang, et al. Allele quantification using molecular inversion probes (MIP). Nucleic Acids Research. 2005; 33(21); e183 (14 pages).
Wapner, et al. First-trimester screening for trisomies 21 and 18. N. Engl. J. Med. 2003; 349:1405-1413.
Warren, et al. Transcription factor profiling in individual hematopoietic progenitors by digital RT-PCR. PNAS. Nov. 21, 2006; 103(47):17807-17812.
Washizu, et al. Handling Biological Cells Utilizing a Fluid Integrated Circuit. IEEE Industry Applications Society Annual Meeting Presentations, Oct. 2-7, 1988;: 1735-40.
Washizu, et al. Handling Biological Cells Utilizing a Fluid Integrated Circuit. IEEE Transactions of Industry Applications. 1990; 26: 352-8.
Weigl, et al. Microfluidic Diffusion-Based Separation and Detection. Science. 1999; pp. 346-347.
Williams, et al. Comparison of cell separation methods to entrich the proportion of fetal cells in material blood samples. The American Journal of Human Genetics. Oct. 1992. Supplemental to vol. 51, No. 4: 1049.
Xiong, et al. "A simple, rapid, high-fidelity and cost-effective PCR-based two-step DNA synthesis method for long gene sequences," Nucleic Acids Research, Apr. 19, 2004, vol. 32, No. 12, e98.
Yang, et al. Prenatal diagnosis of trisomy 21 with fetal cells i maternal blood using comparative genomic hybridization. Fetal Diagn Ther. 2006; 21:125-133.
Yang, et al. Rapid Prenatal Diagnosis of Trisomy 21 by Real-time Quantitative Polymerase Chain Reaction with Amplification of Small Tandem Repeats and S100B in Chromosome 21. Yonsei Medical Journal, 2005, vol. 46, No. 2, 193-197.
Yu, et al. Objective Aneuploidy Detection for Fetal and Neonatal Screening Using Comparative Genomic Hybridization (CGH). Cytometry. 1997; 28(3): 191-197. (Absbract).
Zavala, et al. Genomic GC content prediction in prokaryotes from a sample of genes. Gene. Sep. 12, 2005;357(2):137-43.
Zhen, et al. Poly-FISH: a technique of repeated hybridizations that improves cytogenetic analysis of fetal cells in maternal blood. Prenat Diagn. 1998; 18(11):1181-5.
Zheng, et al. Fetal cell identifiers: results of microscope slide-based immunocytochemical studies as a function of gestational age and abnormality. Am J Obstet Gynecol. May 1999;180(5):1234-9.
Zimmerman, et al. Novel real-time quantitative PCR test for trisomy 21. Jan. 1, 2002. Clinical Chemistry, American Association for Clinical Chemistry. 48:(2) 362-363.
Zuska, P. Microtechnology Opens Doors to the Universe of Small Space, MD&DI Jan. 1997, p. 131.
Rebecca Sparkes, et al., "New Molecular Techniques for the Prenatal Detection of Chromosomal Aneuploidy," JOGC, Jul. 2008, No. 210, 617-621.
Bernhard Zimmermann, "Molecular Diagnosis in Prenatal Medicine," Ph.D. Thesis, 2004.
Jay Shendure, et al., "Next-generation DNA sequencing", Nature, 2008, 26:1135-1145.
Juliane C. Dohm, et al., "Substantial biases in ultra-short read data sets from high-throughput DNA sequencing", Nucleic Acids Research, 2008, 36: e105 doi: 10.1093\nar\gkn425.
Heng Li, et al., "Mapping short DNA sequencing reads and calling variants using mapping quality scores", Genome Research, 2008, doi: 10.1101/gr.078212.108.
Rossa W. K. Chiu, et al., "Non-invasive prenatal diagnosis of fetal chromosomal aneuploidy by massively parallel genomic sequencing of DNA in maternal plasma", PNAS, 2008, 105: 20458-20463.
Melissa J. Fullwood, et al., "Next-generation DNA sequencing of paired-end tags (PET) for transcriptome and genome analyses", Genome Research, 2009, 19: 521-532.
Y.M. Dennis Lo, et al., "Digital PCR for the molecular detection of fetal chromosomal aneuploidy," PNAS, Aug. 7, 2007, vol. 104, No. 32, 13116-13121.
Yuk-Ming Dennis Lo, "Noninvasive prenatal detection of fetal chromosomal aneuploidies by maternal plasma nucleic acid analysis: a review of the current state of the art," BJOG, 2009, vol. 116, 152-157.
H. Christina Fan, et al., "Noninvasive diagnosis of fetal aneuploidy by shotgun sequencing DNA from maternal blood," PNAS, Oct. 21, 2008, vol. 105, 16266-16271.
Fiona M. F. Lun, et al., "Microfluidics Digital PCR Reveals a Higher than Expected Fraction of Fetal DNA in Maternal Plasma," Clinical Chemistry, 2008, vol. 54, No. 10, 1664-1672.
Richard A. White III, et al., "Digital PCR provides sensitive and absolute calibration for high throughput sequencing," BMC GENOMICS, Mar. 19, 2009, 10:116.
Frank Diehl, et al., "Digital quantification of mutant DNA in cancer patients," Curr Opin Oncol, 2007, 19:36-42.
Y-M. D. Lo, et al., "Prenatal Sex Determination by DNA Amplification from Maternal Peripheral Blood," The Lancet, Dec. 9, 1989, 1363-1365.
Y-M. D. Lo, et al., "Detection of fetal RhD sequence from peripheral blood of sensitized RhD-negative pregnant women," British Journal of Haematology, 1994, vol. 87, 658-660.
Y. M. Dennis Lo, et al., "Presence of fetal DNA in maternal plasma and serum," The Lancet, Aug. 16, 1997, vol. 350, 485-487.
Jouni Uitto, et al., "Probing the fetal genome: progress in non-invasive prenatal diagnosis," Trends in Molecular Medicine, Aug. 2003, vol. 9, No. 8, 339-343.
Sinuhe Hahn, et al., "Prenatal Diagnosis Using Fetal Cells and Cell-Free Fetal DNA in Maternal Blood: What is Currently Feasible?" Clinical Obstetrics and Gynecology, Sep. 2002, vol. 45, No. 3, 649-656.
Barbara Pertl, et al., "Fetal DNA in Maternal Plasma: Emerging Clinical Applications," Obstetrics and Gynecology, Sep. 2001, vol. 98, No. 3, 483-490.
Leo L.M. Poon, et al., "Circulating fetal DNA in maternal plasma," Clinical Chimica Acta, 2001, vol. 313, 151-155.
Y-M. D. Lo, et al., "Fetal DNA in Maternal Plasma," Ann. N.Y. Acad. Sci, Apr. 2000, vol. 906, 141-147.
Y-M. D. Lo, et al., "Detection of single-copy fetal DNA sequence from maternal blood," The Lancet, Jun. 16, 1990, vol. 335, 1463-1464.
Y.M. Dennis Lo, et al., "Quantitative Analysis of Fetal NA in Maternal Plasma and Serum: Implications for Noninvasive Prenatal Diagnosis," Am J. Hum. Genet., 1998, vol. 62, 768-775.
Rossa W.K. Chiu, et al., "Effects of Blood-Processing Protocols on Fetal and Total DNA Quantification in Maternal Plasma," Clinical Chemistry, 2001, vol. 47, No. 9, 1607-1613.
Enders K.O. Ng, et al., "The Concentration of Circulating Corticotropin-releasing Hormone mRNA in Maternal Plasma Is Increased in Preeclampsia," Clinical Chemistry, 2003, vol. 49, No. 5, 727-731.
Ido Braslavsky, et al., "Sequence information can be obtained from single DNA molecules," PNAS, Apr. 2003, vol. 100, No. 7, 3960-3964.
Jun Zhu, et al., "Single Molecule Profiling of Alternative Pre-mRNA Splicing," Science, Aug. 2003, vol. 301, 836-838.
Devin Dressman, et al., "Transforming single DNA molecules into fluorescent magnetic particles for detection and enumeration of genetic variations," PNAS, Jul. 2003, vol. 100, No. 15, 8817-8822.

Eugene Y. Chan, et al., "DNA Mapping Using Microfluidic Stretching and Single-Molecule Detection of Fluorescent Site-Specific Tags," Genome Research, 2004, vol. 14, 1137-1146.
Jong Wook Hong, et al., "Molecular biology on a microfluidic chip," Journal of Physics: Condensed Matter, 2006, vol. 18, S691-S701.
Elizabeth A. Ottesen, et al., "Microfluidic Digital PCR Enables Multigene Analysis of Individual Environmental Bacteria," Science, Dec. 2006, vol. 314, 1464-1467.
Joshua S. Marcus, et al., "Parallel Picoliter RT-PCR Assays Using Microfluidics," Analytical Chemistry, Feb. 1, 2006, vol. 78, No. 3, 956-958.
Y. M. Dennis Lo, et al., "Plasma placental RNA allelic ratio permits noninvasive prenatal chromosomal aneuploidy detection," Nature Medicine, Jan. 2007, 1-6.
Joshua S. Marcus, et al., "Microfluidic Single-Cell mRNA Isolation and Analysis," American Chemical Society, Mar. 2006, p. A-F.
Y. M. Dennis Lo, et al., "Prenatal diagnosis: progress through plasma nucleic acids," Nature, Jan. 2007, vol. 8, 71-76.
Tetsuya S. Tanaka, et al., "Genome-wide expression profiling of mid-gestation placenta and embryo using a 15,000 mouse developmental cDNA microarray," PNAS, Aug. 2000, vol. 97, No. 16, 9127-9132.
"Separation of RNA & DNA by Gel Filtration Chromatography," Edvotek, 1987, 1-9.
Ying Li, et al., "Size Separation of Circulatory DNA in Maternal Plasma Permits Ready Detection of Fetal DNA Polymorphisms," 2004, Clinical Chemistry, vol. 50, No. 6, 1002-1001.
H. Christina Fan, et al., "Detection of Aneuploidy with Digital Polymerase Chain Reaction," Analytical Chemistry, Oct. 1, 2007, vol. 79, No. 19, 7576-7579.
H. Christina Fan, et al., "Microfluidic digital PCR enables rapid prenatal diagnosis of fetal aneuploidy," American Journal of Obstetrics & Gynecology, May 2009, 543e1-543-e7.

* cited by examiner

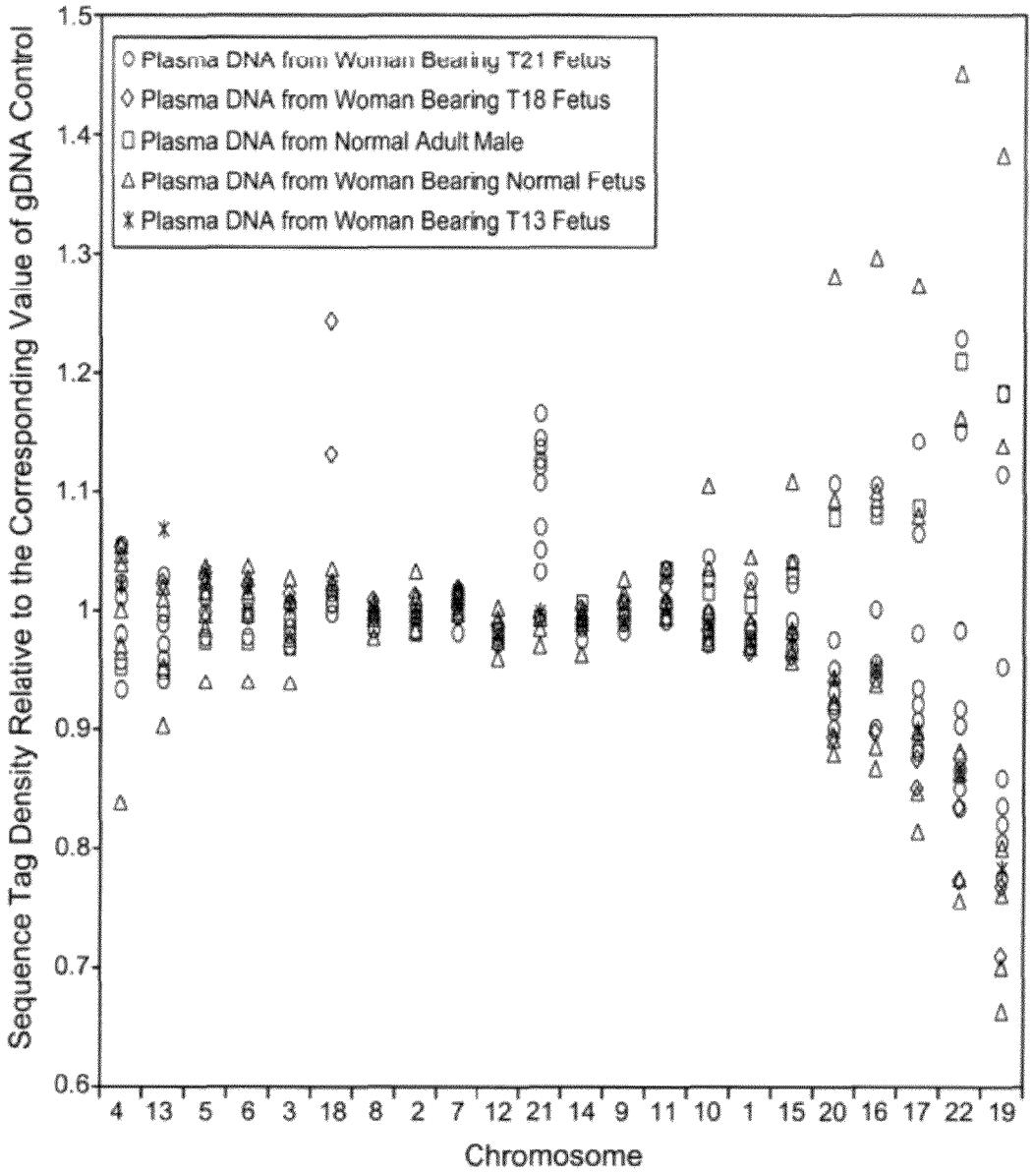

Plasma DNA from Woman Bearing T21 Fetus
Plasma DNA from Woman Bearing T18 Fetus
Plasma DNA from Normal Adult Male
Plasma DNA from Woman Bearing Normal Fetus
Plasma DNA from Woman Bearing T13 Fetus
Sequence Tag Density Relative to the Corresponding Value of gDNA Control
1.5
1.4
1.3
1.2
1.1
1
0.9
0.8
0.7
0.6
4 13 5 6 3 18 8 2 7 12 21 14 9 11 10 1 15 20 16 17 22 19
Chromosome

FIG. 1A

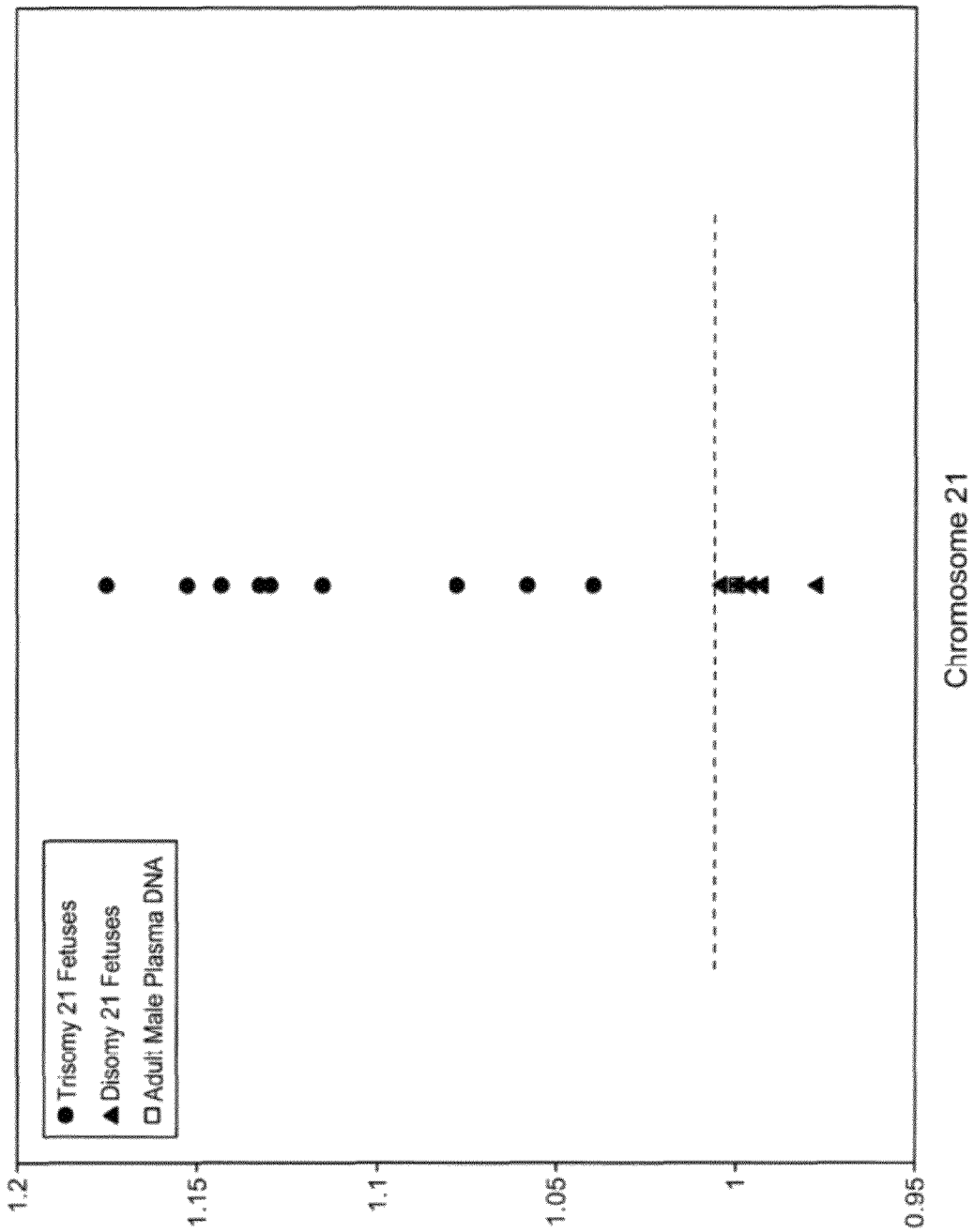

Trisomy 21 Fetuses
Disomy 21 Fetuses
Adult Male Plasma DNA
1.2
1.15
1.1
1.05
1
0.95
Chromosome 21
Sequence Tag Density of Chromosome 21
Relative to the Median Value of Disomy 21 Cases

FIG. 1B

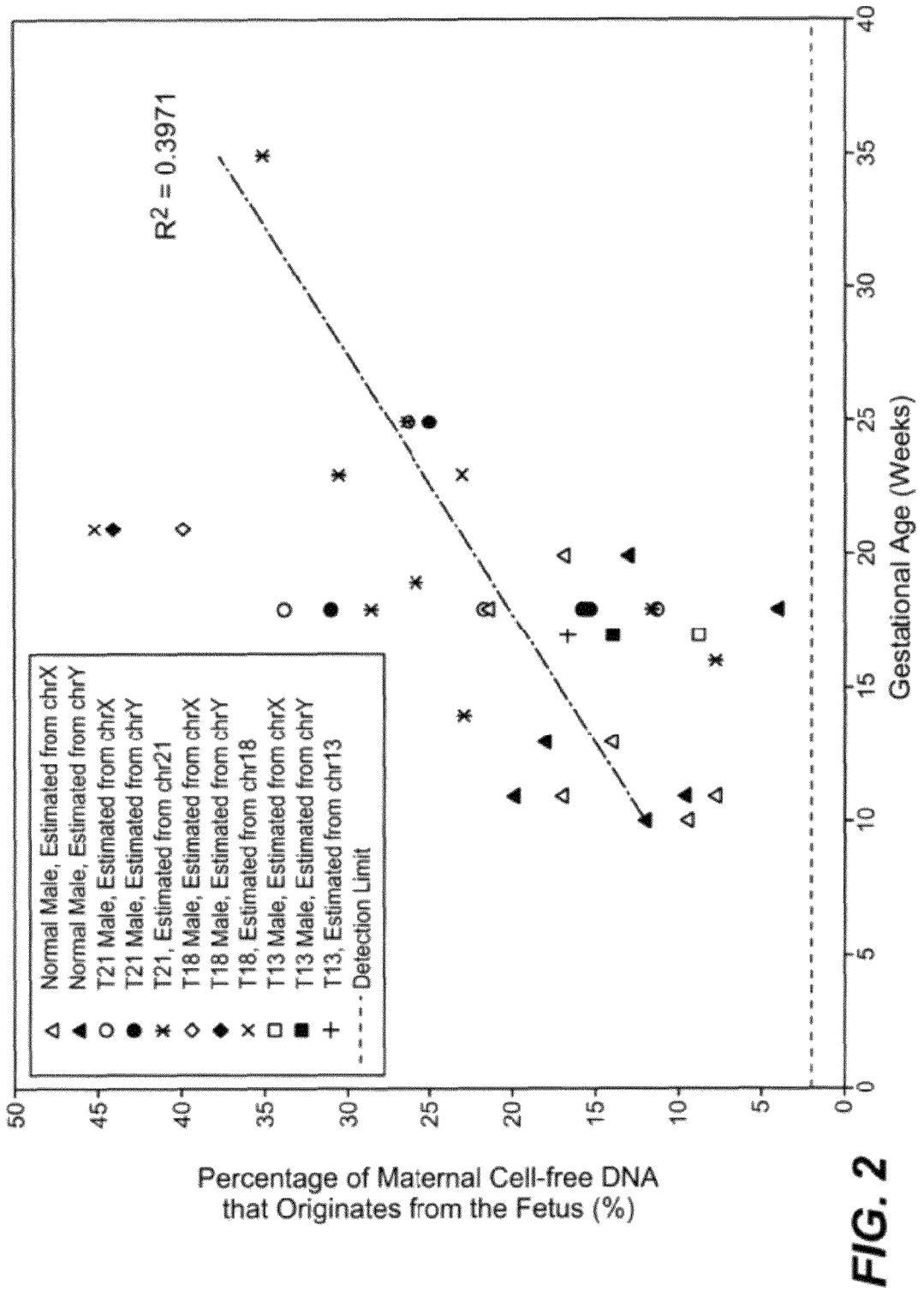

$R^2 = 0.3971$
Normal Male, Estimated from chrX
Normal Male, Estimated from chrY
T21 Male, Estimated from chrX
T21 Male, Estimated from chrY
T21, Estimated from chr21
T18 Male, Estimated from chrX
T18 Male, Estimated from chrY
T18, Estimated from chr18
T13 Male, Estimated from chrX
T13 Male, Estimated from chrY
T13, Estimated from chr13
Detection Limit
Percentage of Maternal Cell-free DNA that Originates from the Fetus (%)
Gestational Age (Weeks)
0
5
10
15
20
25
30
35
40
45
50

FIG. 2

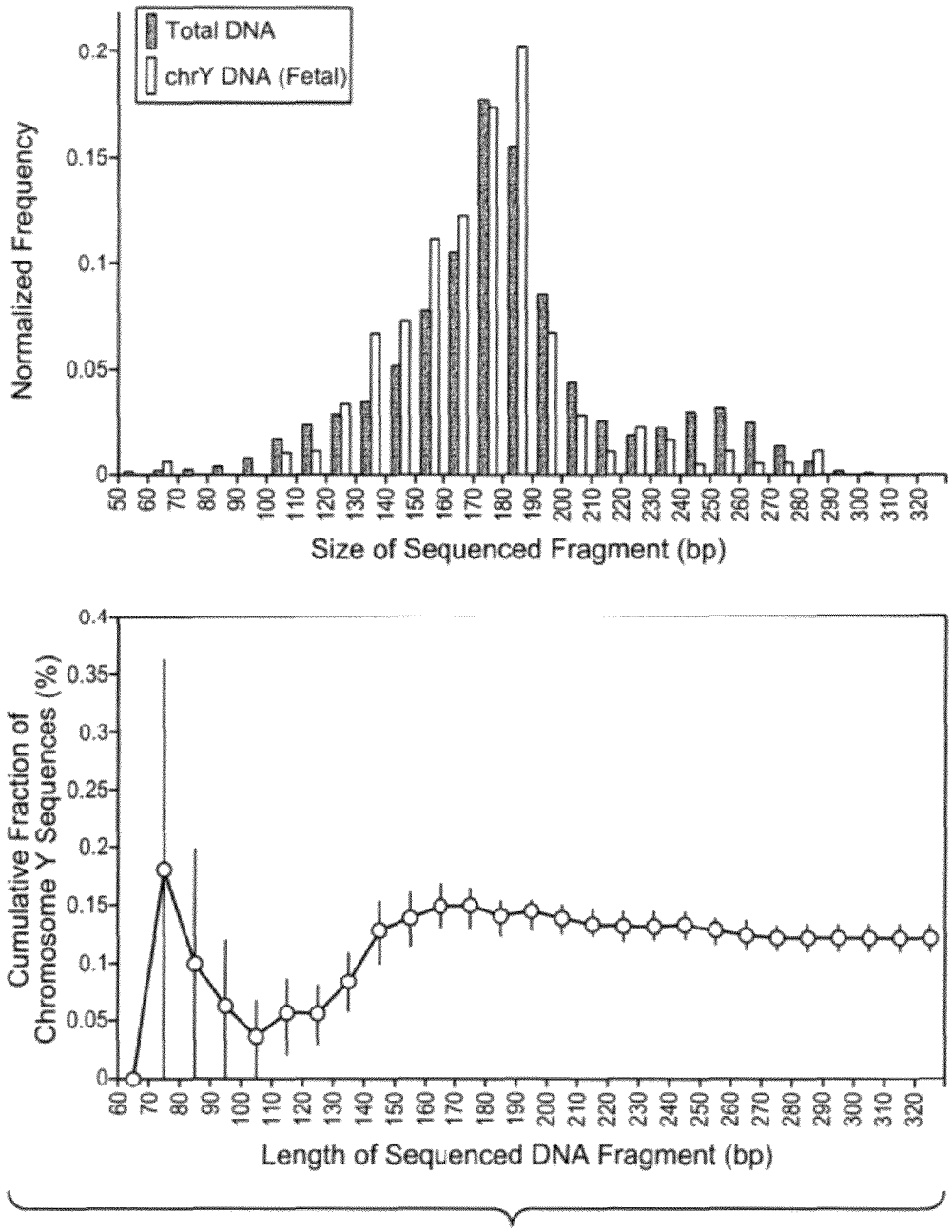

Total DNA
chrY DNA (Fetal)
Normalized Frequency
0
0.05
0.1
0.15
0.2
50 60 70 80 90 100 110 120 130 140 150 160 170 180 190 200 210 220 230 240 250 260 270 280 290 300 310 320
Size of Sequenced Fragment (bp)
Cumulative Fraction of Chromosome Y Sequences (%)
0
0.05
0.1
0.15
0.2
0.25
0.3
0.35
0.4
60 70 80 90 100 110 120 130 140 150 160 170 180 190 200 210 220 230 240 250 260 270 280 290 300 310 320
Length of Sequenced DNA Fragment (bp)

FIG. 3

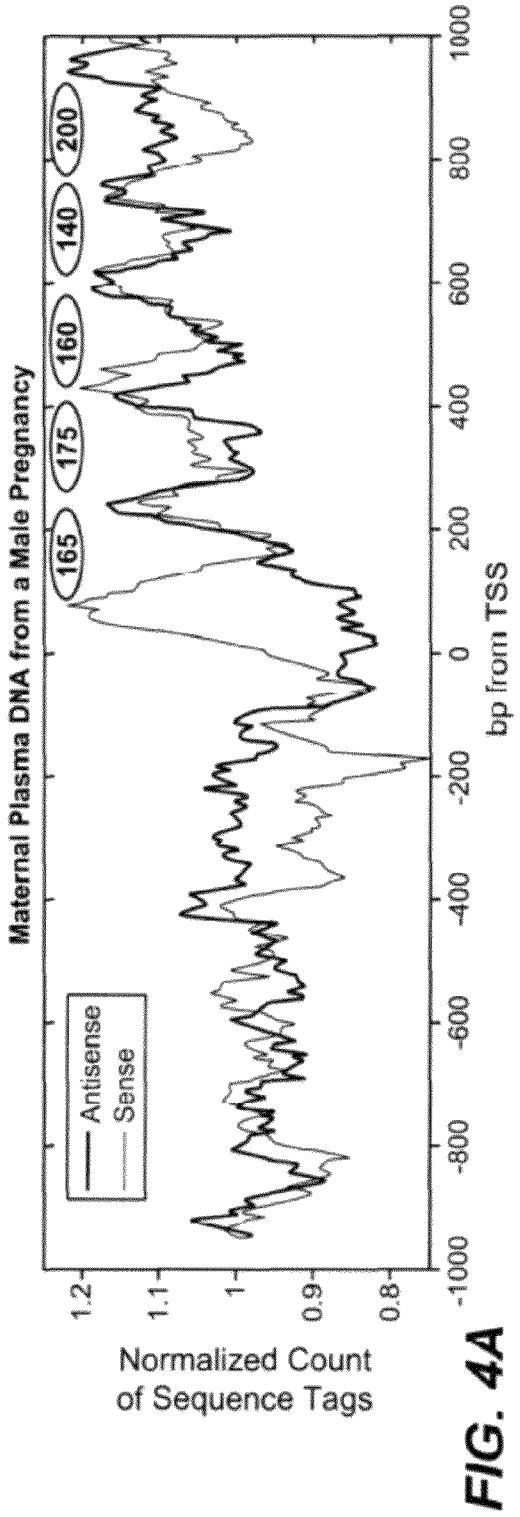

Maternal Plasma DNA from a Male Pregnancy
165
175
160
140
200
Antisense
Sense
1.2
1.1
1
0.9
0.8
Normalized Count of Sequence Tags
-1000
-800
-600
-400
-200
0
200
400
600
800
1000
bp from TSS

FIG. 4A

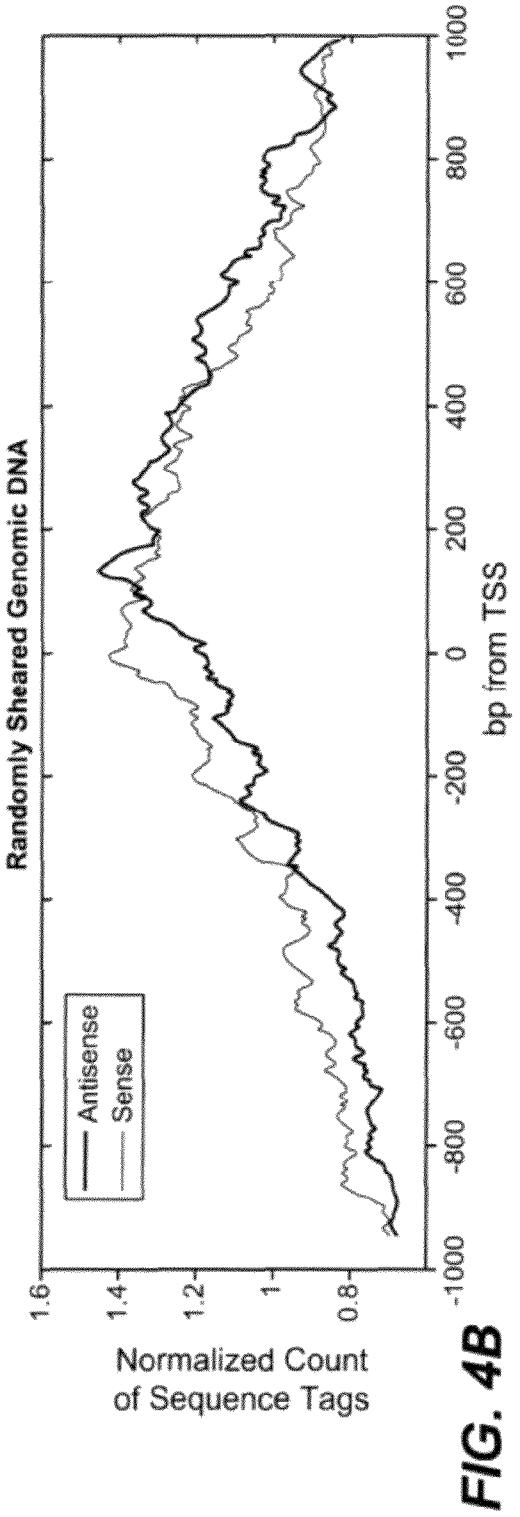

Randomly Sheared Genomic DNA
Antisense
Sense
1.6
1.4
1.2
1
0.8
Normalized Count of Sequence Tags
-1000
-800
-600
-400
-200
0
200
400
600
800
1000
bp from TSS

FIG. 4B

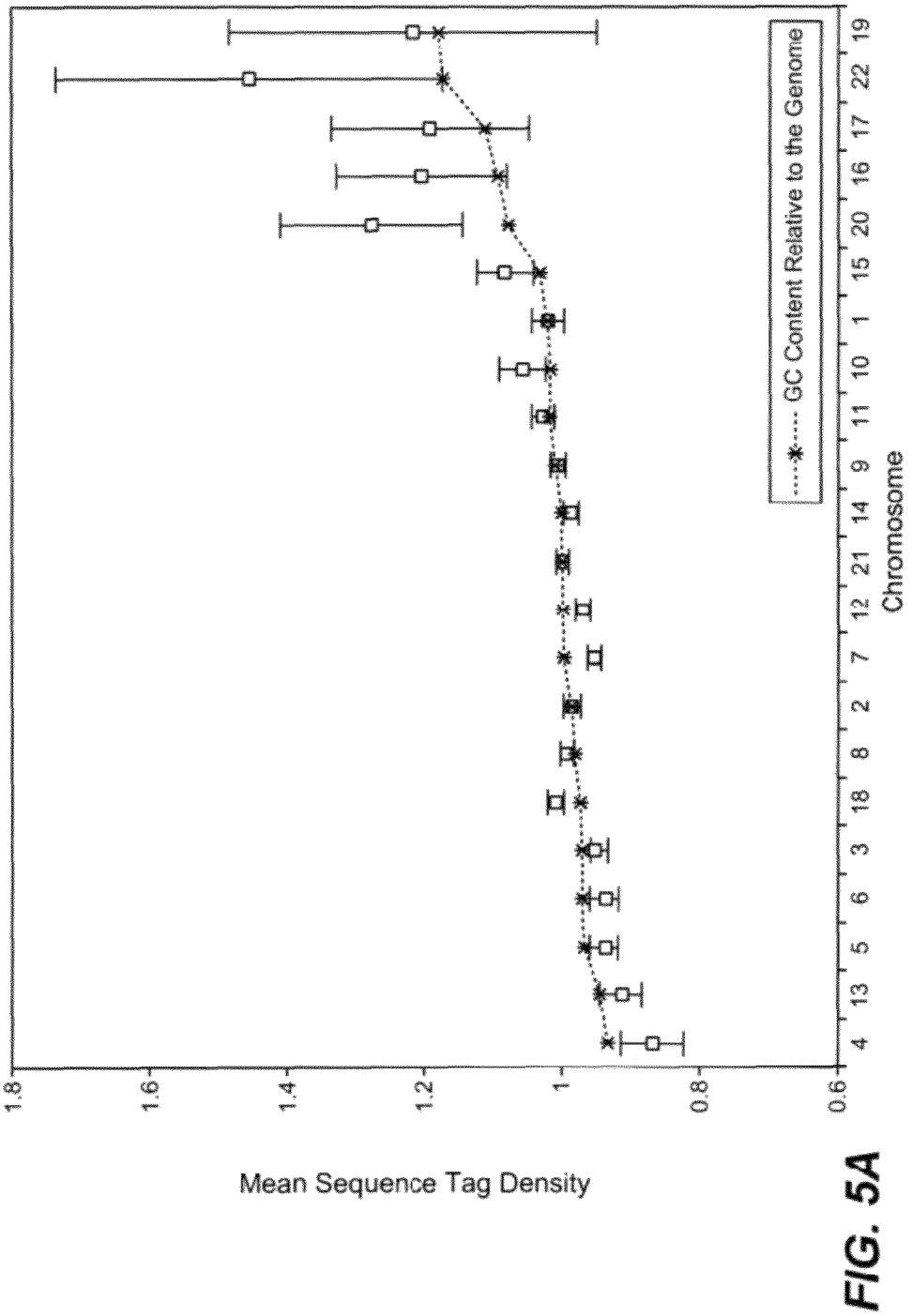

GC Content Relative to the Genome
Chromosome
19
22
17
16
20
15
1
10
11
9
14
21
12
7
2
8
18
3
6
5
13
4
1.8
1.6
1.4
1.2
1
0.8
0.6
Mean Sequence Tag Density

FIG. 5A

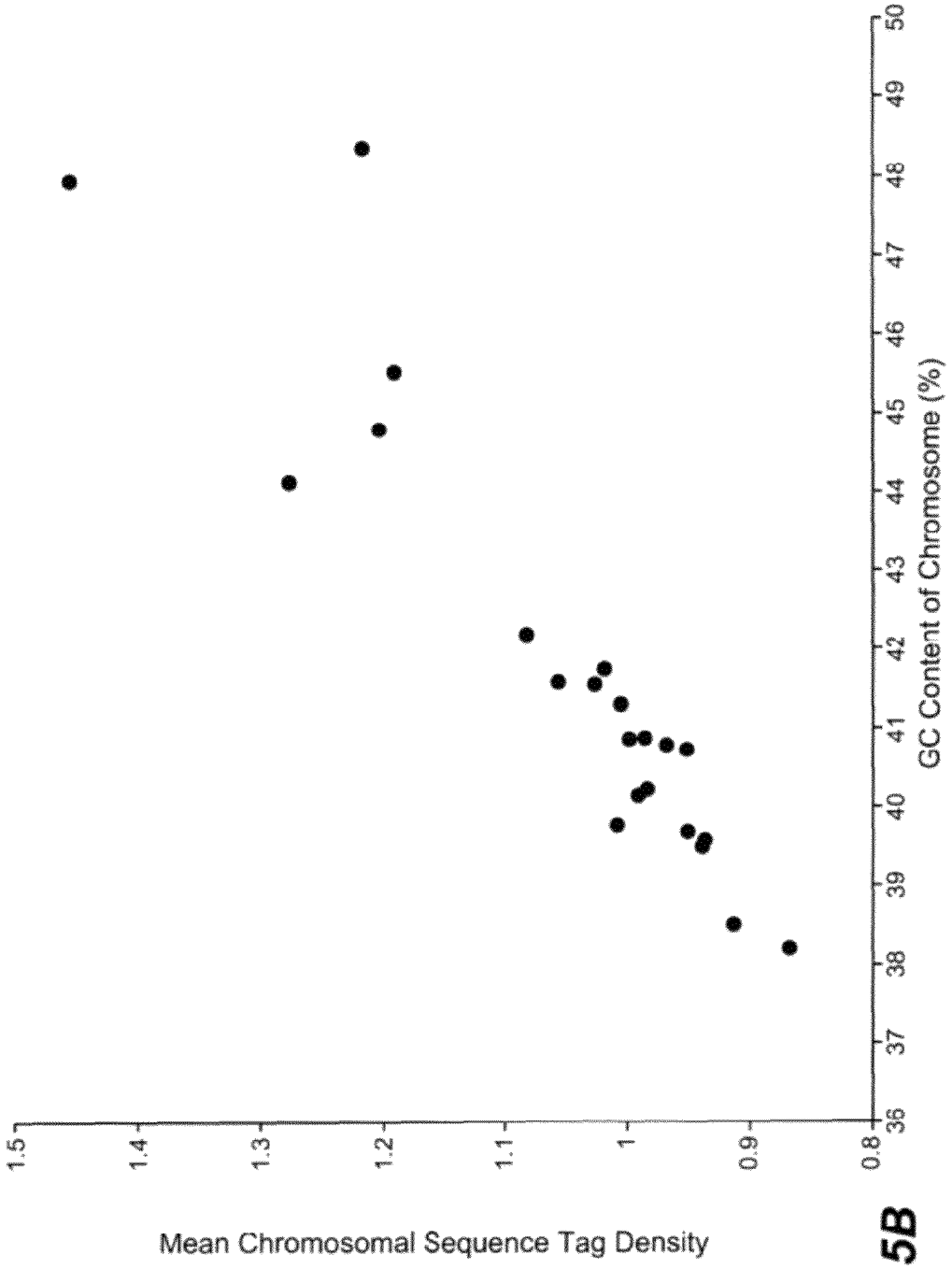

GC Content of Chromosome (%)
50
49
48
47
46
45
44
43
42
41
40
39
38
37
36
Mean Chromosomal Sequence Tag Density
1.5
1.4
1.3
1.2
1.1
1
0.9
0.8

FIG. 5B

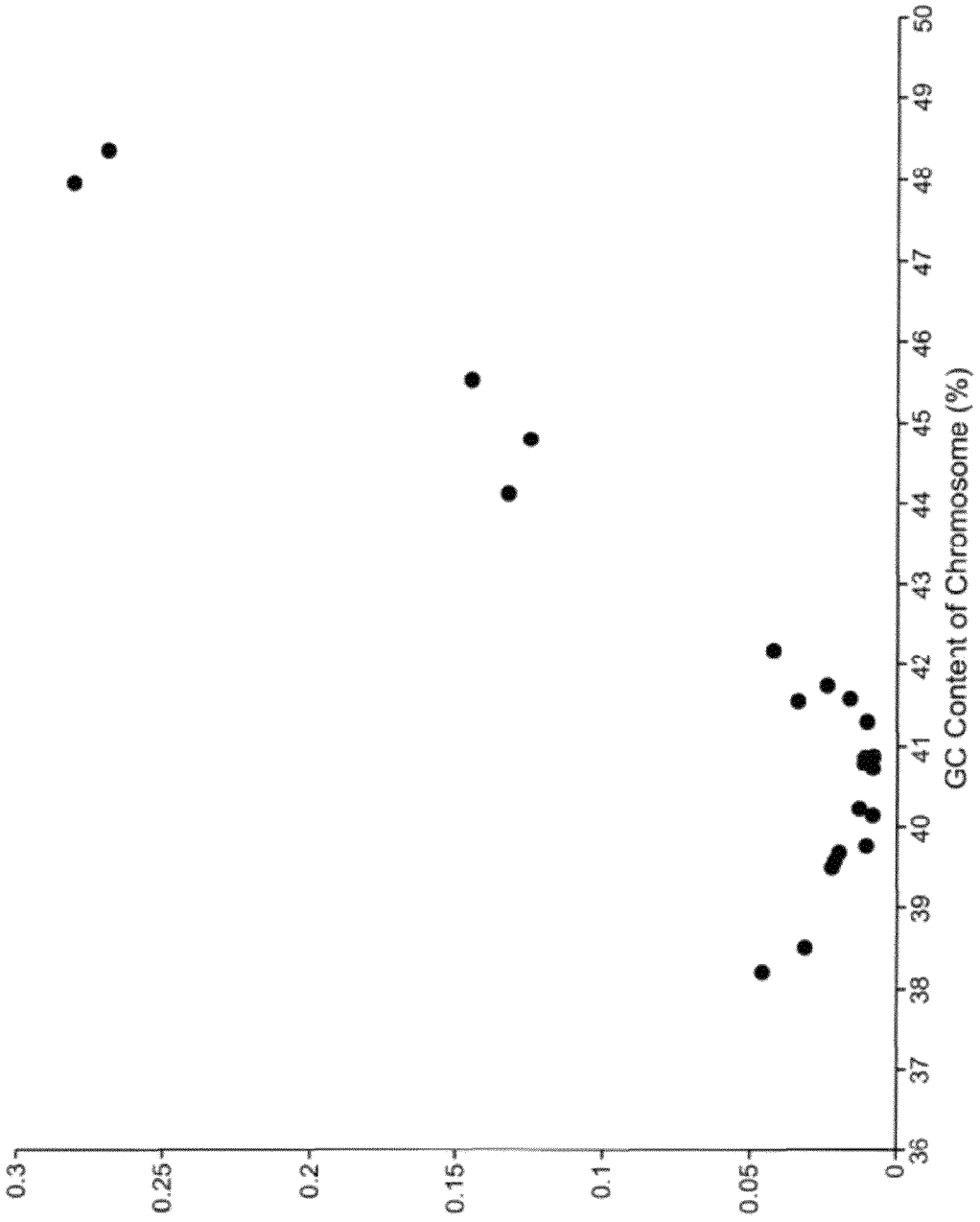

GC Content of Chromosome (%)
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
Standard Deviation of Chromosomal Sequence Tag Density
0
0.05
0.1
0.15
0.2
0.25
0.3

FIG. 5C

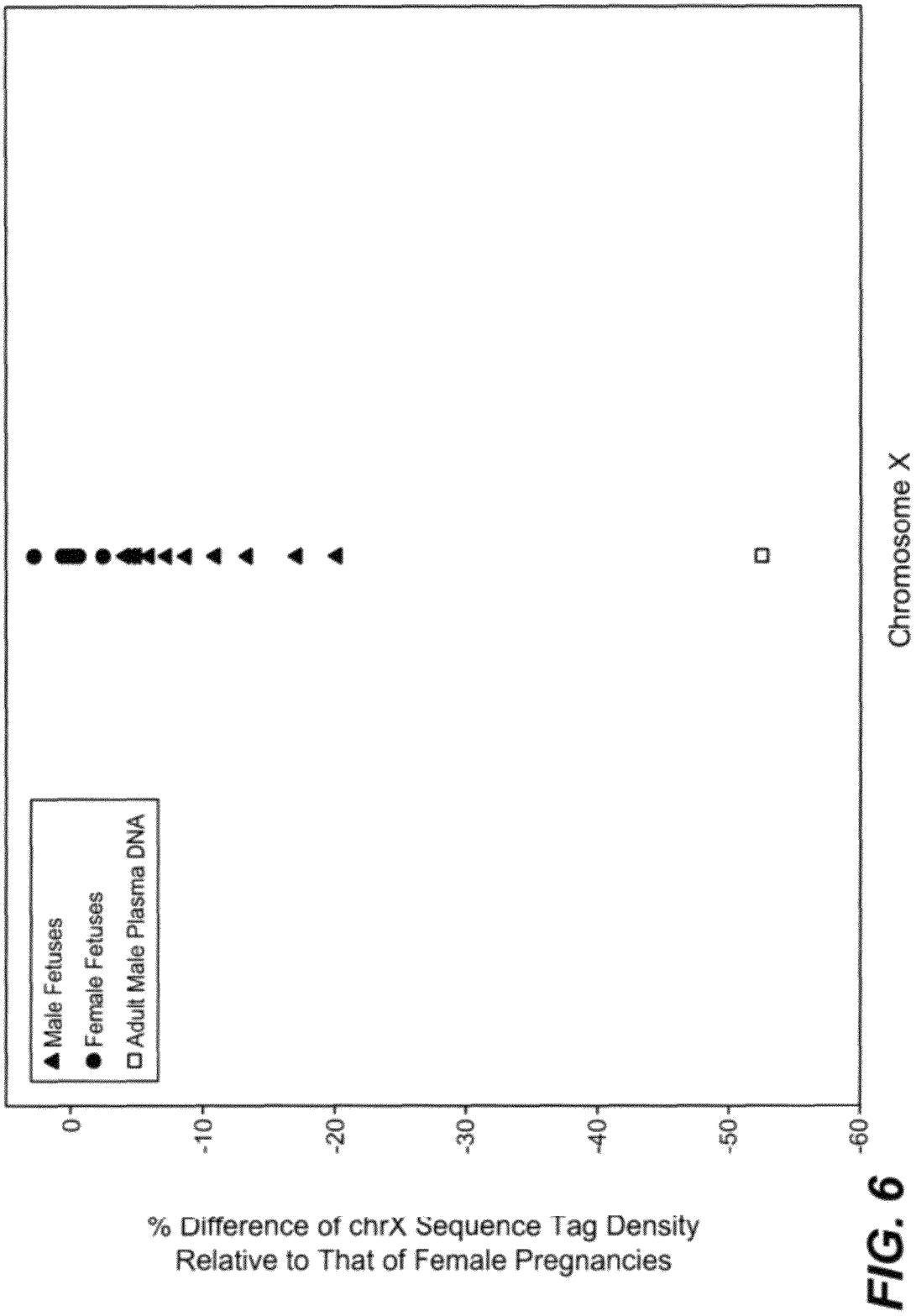

Male Fetuses
Female Fetuses
Adult Male Plasma DNA
0
-10
-20
-30
-40
-50
-60
Chromosome X
% Difference of chrX Sequence Tag Density
Relative to That of Female Pregnancies

FIG. 6

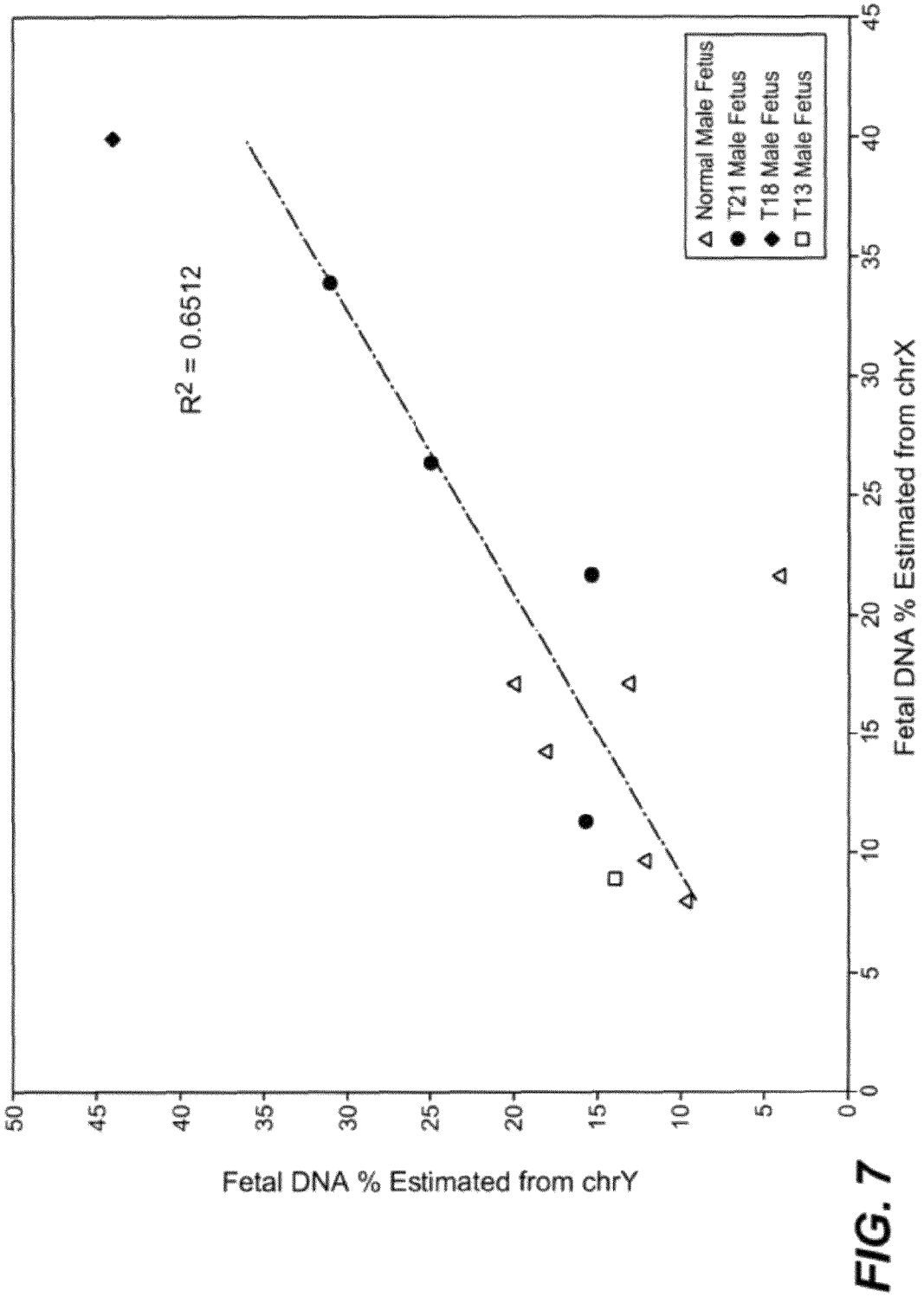

Normal Male Fetus
T21 Male Fetus
T18 Male Fetus
T13 Male Fetus
$R^2 = 0.6512$
Fetal DNA % Estimated from chrY
Fetal DNA % Estimated from chrX
0
5
10
15
20
25
30
35
40
45
50

FIG. 7

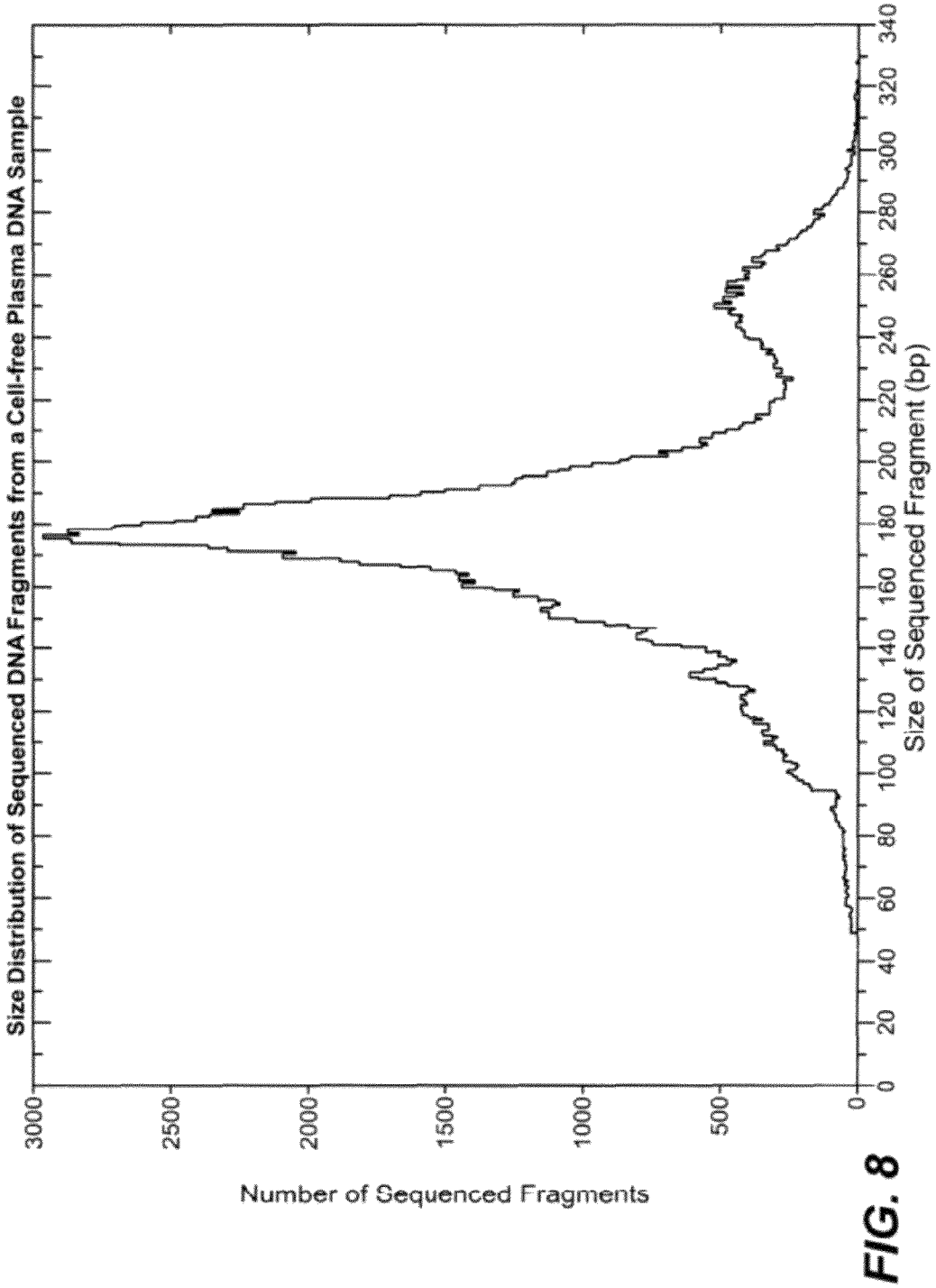

Size Distribution of Sequenced DNA Fragments from a Cell-free Plasma DNA Sample
Number of Sequenced Fragments
3000
2500
2000
1500
1000
500
0
0
20
40
60
80
100
120
140
160
180
200
220
240
260
280
300
320
340
Size of Sequenced Fragment (bp)

FIG. 8

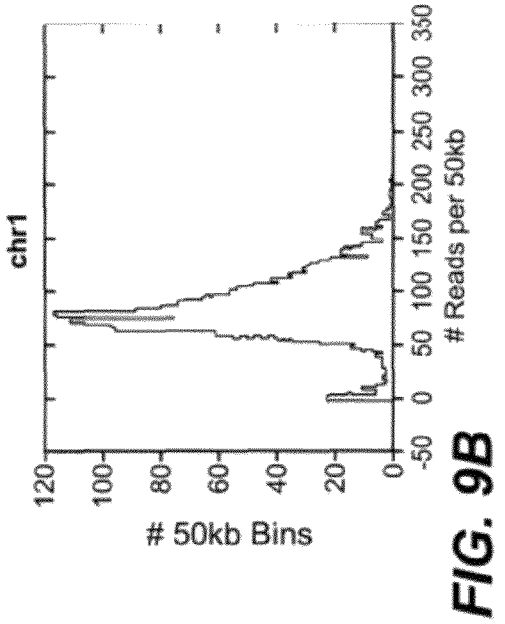


FIG. 9B

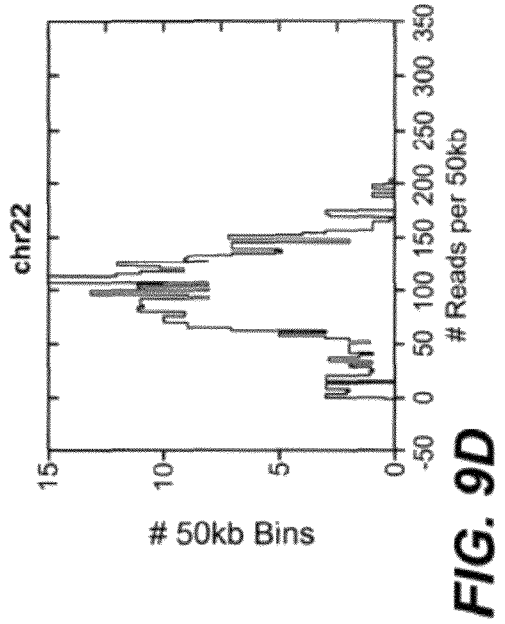


FIG. 9D

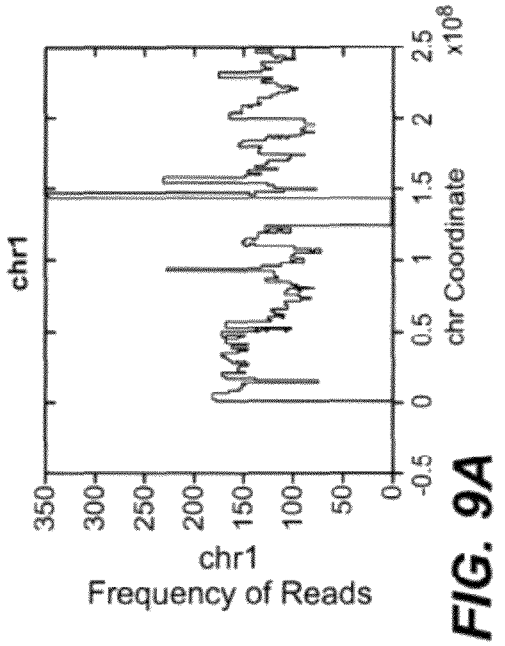


FIG. 9A

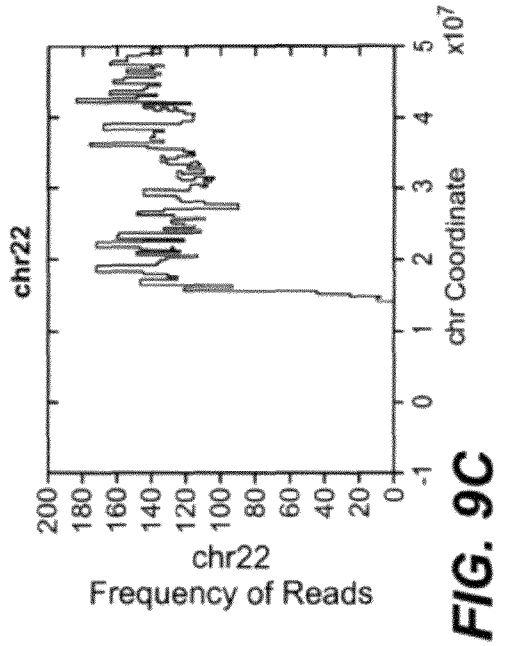


FIG. 9C

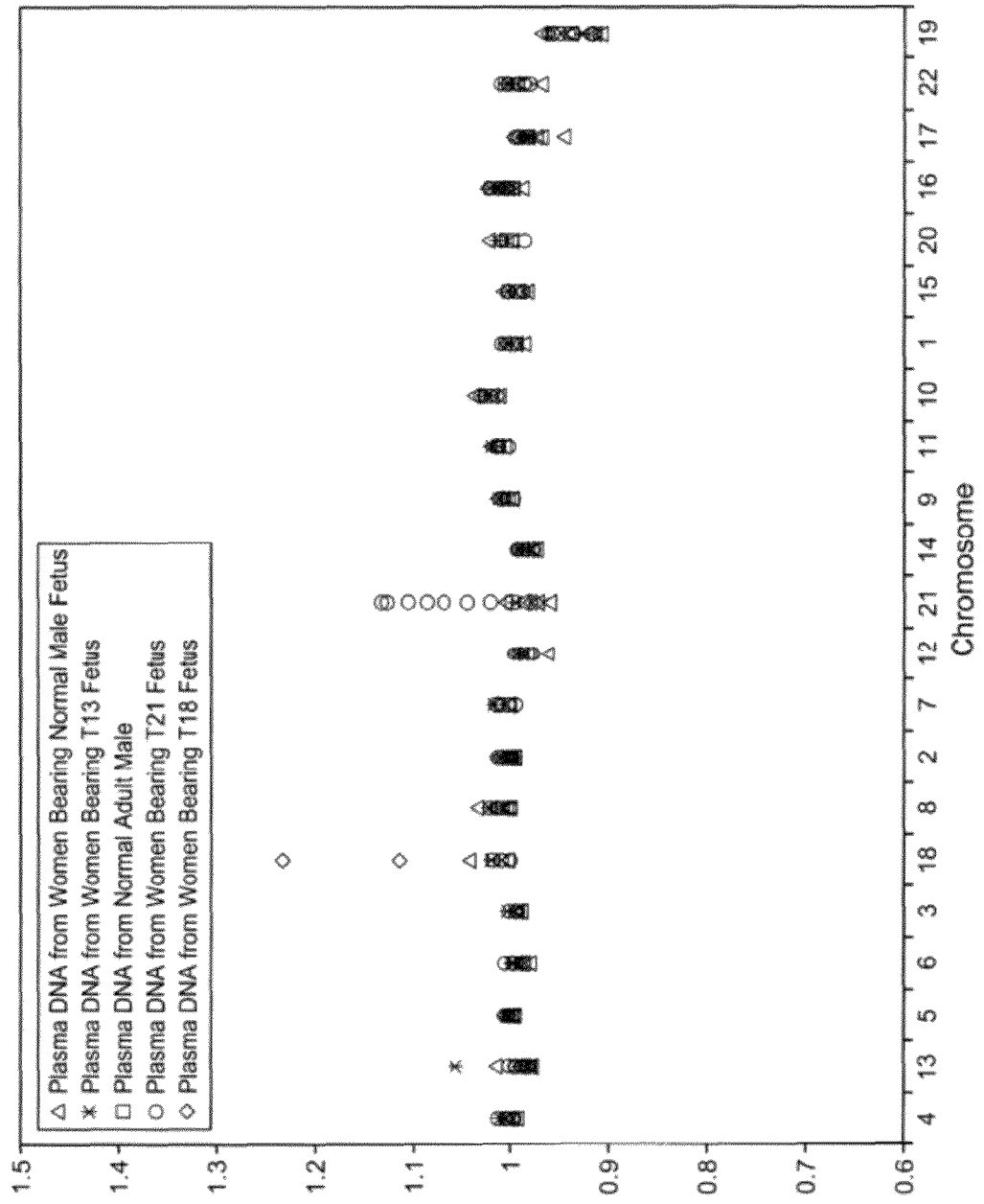

Plasma DNA from Women Bearing Normal Male Fetus
Plasma DNA from Women Bearing T13 Fetus
Plasma DNA from Normal Adult Male
Plasma DNA from Women Bearing T21 Fetus
Plasma DNA from Women Bearing T18 Fetus
1.5
1.4
1.3
1.2
1.1
1
0.9
0.8
0.7
0.6
4 13 5 6 3 18 8 2 7 12 21 14 9 11 10 1 15 20 16 17 22 19
Chromosome
Sequence Tag Density Relative to the Corresponding Value of gDNA Control

FIG. 10

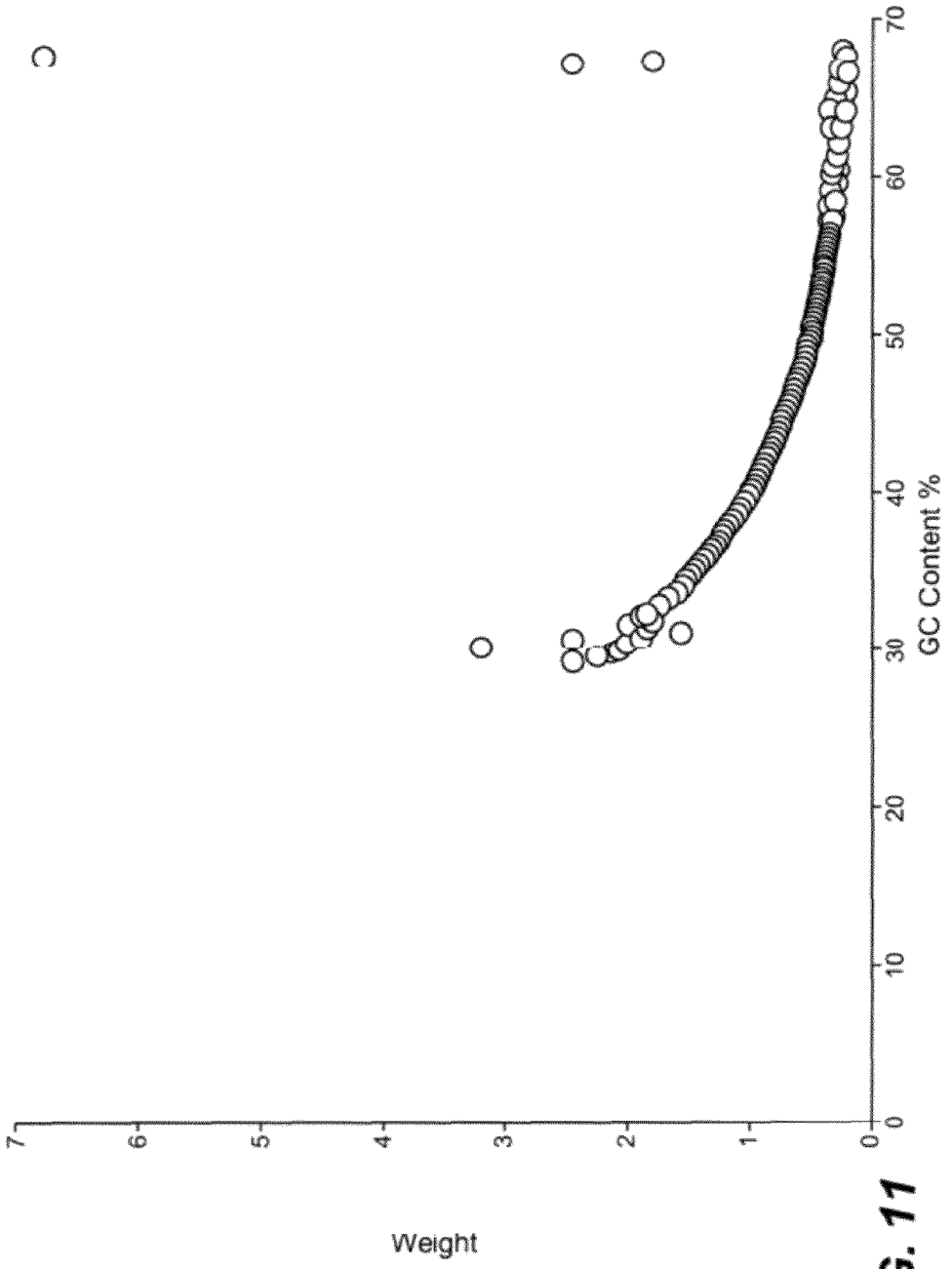


FIG. 11

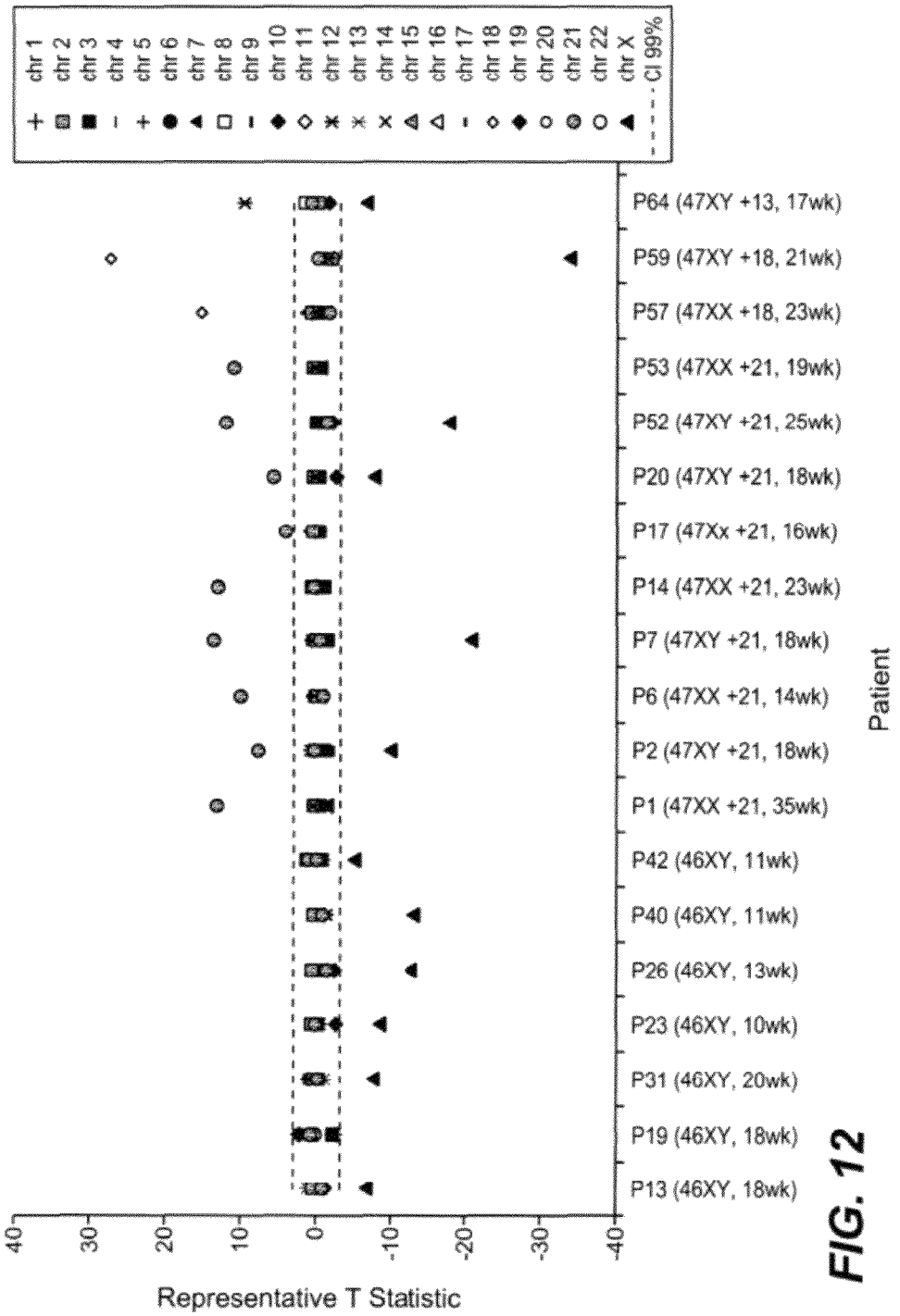

chr 1
chr 2
chr 3
chr 4
chr 5
chr 6
chr 7
chr 8
chr 9
chr 10
chr 11
chr 12
chr 13
chr 14
chr 15
chr 16
chr 17
chr 18
chr 19
chr 20
chr 21
chr 22
chr X
CI 99%
P64 (47XY +13, 17wk)
P59 (47XY +18, 21wk)
P57 (47XX +18, 23wk)
P53 (47XX +21, 19wk)
P52 (47XY +21, 25wk)
P20 (47XY +21, 18wk)
P17 (47Xx +21, 16wk)
P14 (47XX +21, 23wk)
P7 (47XY +21, 18wk)
P6 (47XX +21, 14wk)
P2 (47XY +21, 18wk)
P1 (47XX +21, 35wk)
P42 (46XY, 11wk)
P40 (46XY, 11wk)
P26 (46XY, 13wk)
P23 (46XY, 10wk)
P31 (46XY, 20wk)
P19 (46XY, 18wk)
P13 (46XY, 18wk)
Patient
40
30
20
10
0
-10
-20
-30
-40
Representative T Statistic

FIG. 12

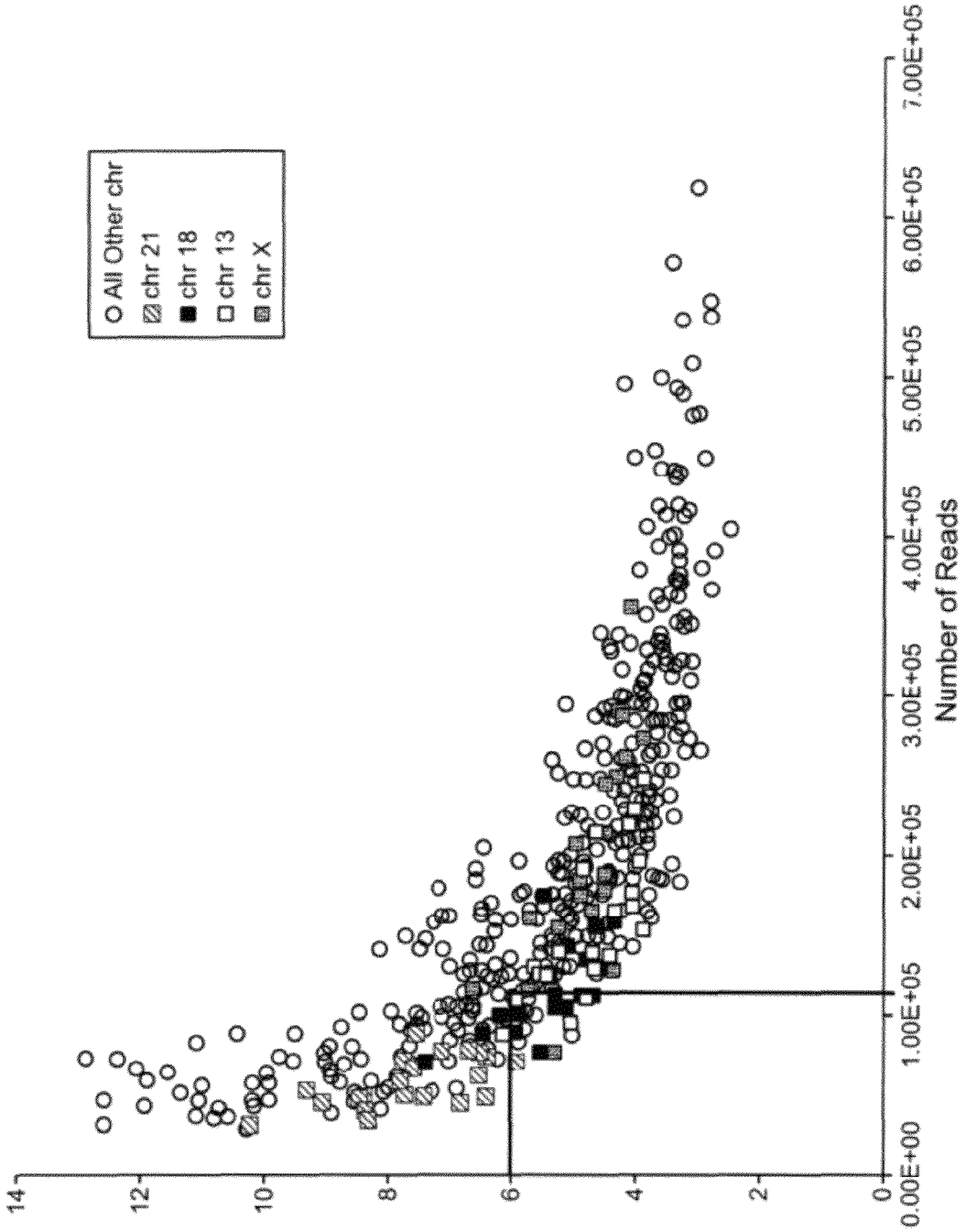

All Other chr
chr 21
chr 18
chr 13
chr X
0.00E+00
1.00E+05
2.00E+05
3.00E+05
4.00E+05
5.00E+05
6.00E+05
7.00E+05
Number of Reads
14
12
10
8
6
4
2
0
Minimum Fetal DNA% of Which Over- or under-representation of Chromosome Could be Detected with 99.9% CI

FIG. 13

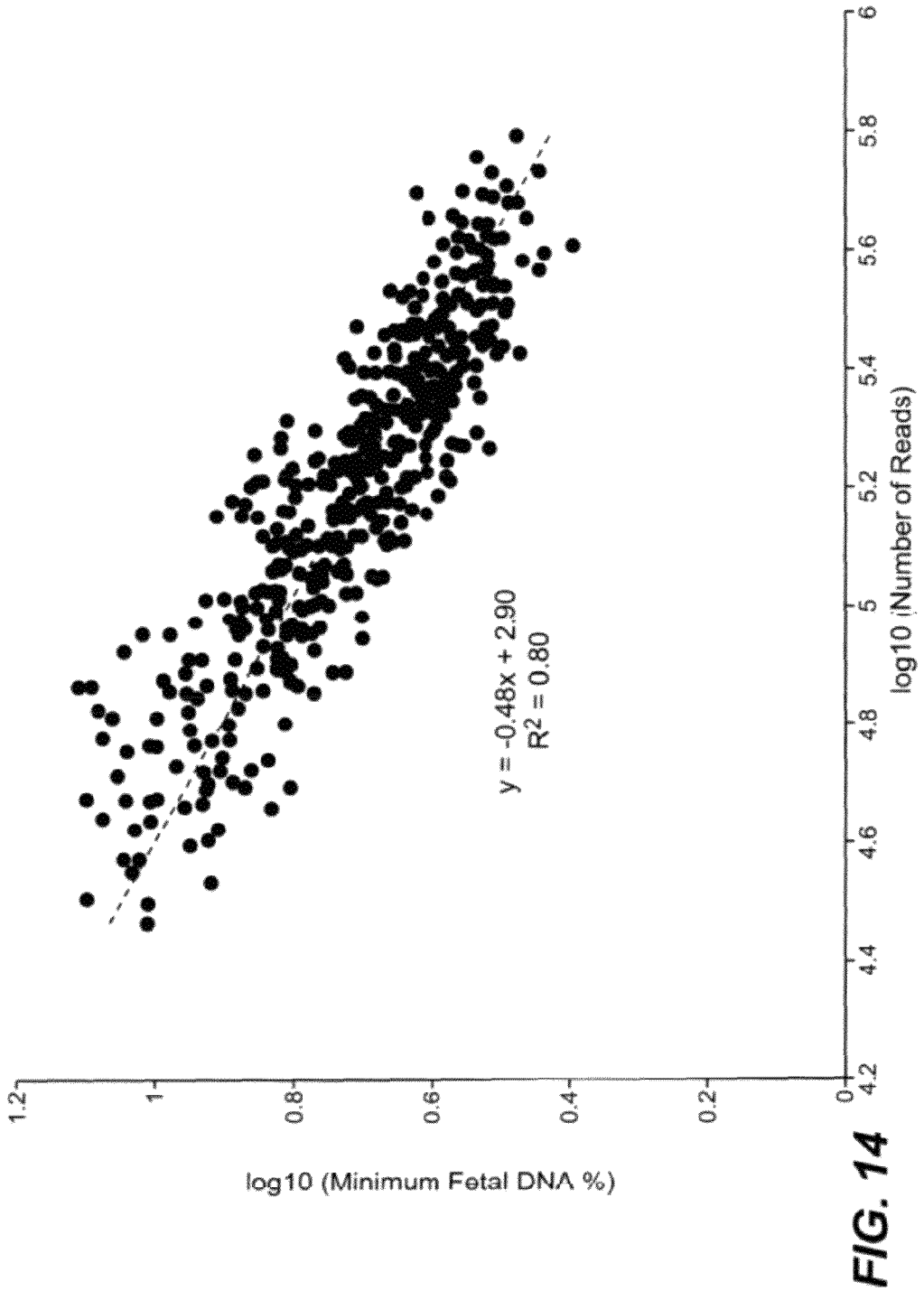

y = -0.48x + 2.90
R2 = 0.80
log10 (Number of Reads)
log10 (Minimum Fetal DNA %)
6
5.8
5.6
5.4
5.2
5
4.8
4.6
4.4
4.2
0
0.2
0.4
0.6
0.8
1
1.2

FIG. 14

NONINVASIVE DIAGNOSIS OF FETAL ANEUOPLOIDY BY SEQUENCING

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority from U.S. Provisional Patent Application No. 61/098,758, filed on Sep. 20, 2008, and U.S. Utility patent application Ser. No. 12/696,509, which is a divisional of U.S. application Ser. No. 12/560,708, filed Sep. 16, 2009, was filed Jan. 29, 2010, and is now U.S. Pat. No. 8,195,415, both of which are hereby incorporated by reference in their entirety.

STATEMENT OF GOVERNMENTAL SUPPORT

This invention was made with Government support under contract DP1 OD000251 awarded by the National Institutes of Health. The Government has certain rights in this invention.

REFERENCE TO SEQUENCE LISTING, COMPUTER PROGRAM, OR COMPACT DISK

Applicants submit herewith a sequence listing in an ASCII text file (3815_63_5_seq_list.txt), as provided in EFS Legal Framework Notice 20 May 2010, part I-I-1. The file was created Apr. 19, 2012 and contains 2,695 bytes. Applicants incorporate the contents of the sequence listing by reference in its entirety.

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates to the field of molecular diagnostics, and more particularly to the field of prenatal genetic diagnosis.

2. Related Art

Presented below is background information on certain aspects of the present invention as they may relate to technical features referred to in the detailed description, but not necessarily described in detail. That is, certain components of the present invention may be described in greater detail in the materials discussed below. The discussion below should not be construed as an admission as to the relevance of the information to the claimed invention or the prior art effect of the material described.

Fetal aneuploidy and other chromosomal aberrations affect 9 out of 1000 live births (1). The gold standard for diagnosing chromosomal abnormalities is karyotyping of fetal cells obtained via invasive procedures such as chorionic villus sampling and amniocentesis. These procedures impose small but potentially significant risks to both the fetus and the mother (2). Non-invasive screening of fetal aneuploidy using maternal serum markers and ultrasound are available but have limited reliability (3-5). There is therefore a desire to develop non-invasive genetic tests for fetal chromosomal abnormalities.

Since the discovery of intact fetal cells in maternal blood, there has been intense interest in trying to use them as a diagnostic window into fetal genetics (6-9). While this has not yet moved into practical application (10), the later discovery that significant amounts of cell-free fetal nucleic acids also exist in maternal circulation has led to the development of new non-invasive prenatal genetic tests for a variety of traits (11, 12). However, measuring aneuploidy remains challenging due to the high background of maternal DNA; fetal DNA often constitutes <10% of total DNA in maternal cell-free plasma (13).

Recently developed methods for aneuploidy rely on detection focus on allelic variation between the mother and the fetus. Lo et al. demonstrated that allelic ratios of placental specific mRNA in maternal plasma could be used to detect trisomy 21 in certain populations (14).

Similarly, they also showed the use of allelic ratios of imprinted genes in maternal plasma DNA to diagnose trisomy 18 (15). Dhallan et al. used fetal specific alleles in maternal plasma DNA to detect trisomy 21 (16). However, these methods are limited to specific populations because they depend on the presence of genetic polymorphisms at specific loci. We and others argued that it should be possible in principle to use digital PCR to create a universal, polymorphism independent test for fetal aneuploidy using maternal plasma DNA (17-19).

An alternative method to achieve digital quantification of DNA is direct shotgun sequencing followed by mapping to the chromosome of origin and enumeration of fragments per chromosome. Recent advances in DNA sequencing technology allow massively parallel sequencing (20), producing tens of millions of short sequence tags in a single run and enabling a deeper sampling than can be achieved by digital PCR. As is known in the art, the term "sequence tag" refers to a relatively short (e.g., 15-100) nucleic acid sequence that can be used to identify a certain larger sequence, e.g., be mapped to a chromosome or genomic region or gene. These can be ESTs or expressed sequence tags obtained from mRNA.

Specific Patents and Publications

Science 309:1476 (2 Sep. 2005) News Focus "An Earlier Look at Baby's Genes" describes attempts to develop tests for Down Syndrome using maternal blood. Early attempts to detect Down Syndrome using fetal cells from maternal blood were called "just modestly encouraging." The report also describes work by Dennis Lo to detect the Rh gene in a fetus where it is absent in the mother. Other mutations passed on from the father have reportedly been detected as well, such as cystic fibrosis, beta-thalassemia, a type of dwarfism and Huntington's disease. However, these results have not always been reproducible.

Venter et al., "The sequence of the human genome," *Science,* 2001 Feb. 16; 291(5507):1304-51 discloses the sequence of the human genome, which information is publicly available from NCBI. Another reference genomic sequence is a current NCBI build as obtained from the UCSC genome gateway.

Wheeler et al., "The complete genome of an individual by massively parallel DNA sequencing," *Nature,* 2008 Apr. 17; 452(7189):872-6 discloses the DNA sequence of a diploid genome of a single individual, James D. Watson, sequenced to 7.4-fold redundancy in two months using massively parallel sequencing in picoliter-size reaction vessels. Comparison of the sequence to the reference genome led to the identification of 3.3 million single nucleotide polymorphisms, of which 10,654 cause amino-acid substitution within the coding sequence.

Quake et al., US 2007/0202525 entitled "Non-invasive fetal genetic screening by digital analysis," published Aug. 30, 2007, discloses a process in which maternal blood containing fetal DNA is diluted to a nominal value of approximately 0.5 genome equivalent of DNA per reaction sample.

Chiu et al., "Noninvasive prenatal diagnosis of fetal chromosomal aneuploidy by massively parallel genomic DNA sequencing of DNA in maternal plasma," *Proc. Natl. Acad. Sci.* 105(51):20458-20463 (Dec. 23, 2008) discloses a method for determining fetal aneuploidy using massively

parallel sequencing. Disease status determination (aneuploidy) was made by calculating a "z score." Z scores were compared with reference values, from a population restricted to euploid male fetuses. The authors noted in passing that G/C content affected the coefficient of variation.

Lo et al., "Diagnosing Fetal Chromosomal Aneuploidy Using Massively Parallel Genomic Sequencing," US 2009/0029377, published Jan. 29, 2009, discloses a method in which respective amounts of a clinically-relevant chromosome and of background chromosomes are determined from results of massively parallel sequencing. It was found that the percentage representation of sequences mapped to chromosome 21 is higher in a pregnant woman carrying a trisomy 21 fetus when compared with a pregnant woman carrying a normal fetus. For the four pregnant women each carrying a euploid fetus, a mean of 1.345% of their plasma DNA sequences were aligned to chromosome 21.

Lo et al., Determining a Nucleic Acid Sequence Imbalance," US 2009/0087847 published Apr. 2, 2009, discloses a method for determining whether a nucleic acid sequence imbalance exists, such as an aneuploidy, the method comprising deriving a first cutoff value from an average concentration of a reference nucleic acid sequence in each of a plurality of reactions, wherein the reference nucleic acid sequence is either the clinically relevant nucleic acid sequence or the background nucleic acid sequence; comparing the parameter to the first cutoff value; and based on the comparison, determining a classification of whether a nucleic acid sequence imbalance exists.

BRIEF SUMMARY OF THE INVENTION

The following brief summary is not intended to include all features and aspects of the present invention, nor does it imply that the invention must include all features and aspects discussed in this summary.

The present invention comprises a method for analyzing a maternal sample, e.g., from peripheral blood. It is not invasive into the fetal space, as is amniocentesis or chorionic villi sampling. In the preferred method, fetal DNA which is present in the maternal plasma is used. The fetal DNA is in one aspect of the invention enriched due to the bias in the method towards shorter DNA fragments, which tend to be fetal DNA. The method is independent of any sequence difference between the maternal and fetal genome. The DNA obtained, preferably from a peripheral blood draw, is a mixture of fetal and maternal DNA. The DNA obtained is at least partially sequenced, in a method which gives a large number of short reads. These short reads act as sequence tags, in that a significant fraction of the reads are sufficiently unique to be mapped to specific chromosomes or chromosomal locations known to exist in the human genome. They are mapped exactly, or may be mapped with one mismatch, as in the examples below. By counting the number of sequence tags mapped to each chromosome (1-22, X and Y), the over- or under-representation of any chromosome or chromosome portion in the mixed DNA contributed by an aneuploid fetus can be detected. This method does not require the sequence differentiation of fetal versus maternal DNA, because the summed contribution of both maternal and fetal sequences in a particular chromosome or chromosome portion will be different as between an intact, diploid chromosome and an aberrant chromosome, i.e., with an extra copy, missing portion or the like. In other words, the method does not rely on a priori sequence information that would distinguish fetal DNA from maternal DNA. The abnormal distribution of a fetal chromosome or portion of a chromosome (i.e., a gross deletion or insertion) may be determined in the present method by enumeration of sequence tags as mapped to different chromosomes. The median count of autosomal values (i.e., number of sequence tags per autosome) is used as a normalization constant to account for differences in total number of sequence tags is used for comparison between samples and between chromosomes The term "chromosome portion" is used herein to denote either an entire chromosome or a significant fragment of a chromosome. For example, moderate Down syndrome has been associated with partial trisomy 21q22.2→qter. By analyzing sequence tag density in predefined subsections of chromosomes (e.g., 10 to 100 kb windows), a normalization constant can be calculated, and chromosomal subsections quantified (e.g., 21q22.2). With large enough sequence tag counts, the present method can be applied to arbitrarily small fractions of fetal DNA. It has been demonstrated to be accurate down to 6% fetal DNA concentration. Exemplified below is the successful use of shotgun sequencing and mapping of DNA to detect fetal trisomy 21 (Down syndrome), trisomy 18 (Edward syndrome), and trisomy 13 (Patau syndrome), carried out non-invasively using cell-free fetal DNA in maternal plasma. This forms the basis of a universal, polymorphism-independent non-invasive diagnostic test for fetal aneuploidy. The sequence data also allowed us to characterize plasma DNA in unprecedented detail, suggesting that it is enriched for nucleosome bound fragments. The method may also be employed so that the sequence data obtained may be further analyzed to obtain information regarding polymorphisms and mutations.

Thus, the present invention comprises, in certain aspects, a method of testing for an abnormal distribution of a specified chromosome portion in a mixed sample of normally and abnormally distributed chromosome portions obtained from a single subject, such as a mixture of fetal and maternal DNA in a maternal plasma sample. One carries out sequence determinations on the DNA fragments in the sample, obtaining sequences from multiple chromosome portions of the mixed sample to obtain a number of sequence tags of sufficient length of determined sequence to be assigned to a chromosome location within a genome and of sufficient number to reflect abnormal distribution. Using a reference sequence, one assigns the sequence tags to their corresponding chromosomes including at least the specified chromosome by comparing the sequence to reference genomic sequence. Often there will be on the order of millions of short sequence tags that are assigned to certain chromosomes, and, importantly, certain positions along the chromosomes. One then may determine a first number of sequence tags mapped to at least one normally distributed chromosome portion and a second number of sequence tags mapped to the specified chromosome portion, both chromosomes being in one mixed sample. The present method also involves correcting for nonuniform distribution sequence tags to different chromosomal portions. This is explained in detail below, where a number of windows of defined length are created along a chromosome, the windows being on the order of kilobases in length, whereby a number of sequence tags will fall into many of the windows and the windows covering each entire chromosome in question, with exceptions for non-informative regions, e.g., centromere regions and repetitive regions. Various average numbers, i.e., median values, are calculated for different windows and compared. By counting sequence tags within a series of predefined windows of equal lengths along different chromosomes, more robust and statistically significant results may be obtained. The present method also involves calculating a

differential between the first number and the second number which is determinative of whether or not the abnormal distribution exists.

In certain aspects, the present invention may comprise a computer programmed to analyze sequence data obtained from a mixture of maternal and fetal chromosomal DNA. Each autosome (chr. 1-22) is computationally segmented into contiguous, non-overlapping windows. (A sliding window could also be used). Each window is of sufficient length to contain a significant number of reads (sequence tags, having about 20-100 bp of sequence) and not still have a number of windows per chromosome. Typically, a window will be between 10 kb and 100 kb, more typically between 40 and 60 kb. There would, then, for example, accordingly be approximately between 3,000 and 100,000 windows per chromosome. Windows may vary widely in the number of sequence tags that they contain, based on location (e.g., near a centromere or repeating region) or G/C content, as explained below. The median (i.e., middle value in the set) count per window for each chromosome is selected; then the median of the autosomal values is used to account for differences in total number of sequence tags obtained for different chromosomes and distinguish interchromosomal variation from sequencing bias from aneuploidy. This mapping method may also be applied to discern partial deletions or insertions in a chromosome. The present method also provides a method for correcting for bias resulting from G/C content. For example, some the Solexa sequencing method was found to produce more sequence tags from fragments with increased G/C content. By assigning a weight to each sequence tag based on the G/C content of a window in which the read falls. The window for GC calculation is preferably smaller than the window for sequence tag density calculation.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** is a scatter plot graph showing sequence tag densities from eighteen samples, having five different genotypes, as indicated in the figure legend. Fetal aneuploidy is detectable by the over-representation of the affected chromosome in maternal blood. FIG. **1**A shows sequence tag density relative to the corresponding value of genomic DNA control; chromosomes are ordered by increasing G/C content. The samples shown as indicated, are plasma from a woman bearing a T21 fetus; plasma from a woman bearing a T18 fetus; plasma from a normal adult male; plasma from a woman bearing a normal fetus; plasma from a woman bearing a T13 fetus. Sequence tag densities vary more with increasing chromosomal G/C content. FIG. **1**B is a detail from FIG. **1**A, showing chromosome 21 sequence tag density relative to the median chromosome 21 sequence tag density of the normal cases. Note that the values of 3 disomy 21 cases overlap at 1.0. The dashed line represents the upper boundary of the 99% confidence interval constructed from all disomy 21 samples. The chromosomes are listed in FIG. **1**A in order of G/C content, from low to high. This figure suggests that one would prefer to use as a reference chromosome in the mixed sample with a mid level of G/C content, as it can be seen that the data there are more tightly grouped. That is, chromosomes 18, 8, 2, 7, 12, 21 (except in suspected Down syndrome), 14, 9, and 11 may be used as the nominal diploid chromosome if looking for a trisomy. FIG. **1**B represents an enlargement of the chromosome 21 data.

FIG. **2** is a scatter plot graph showing fetal DNA fraction and gestational age. The fraction of fetal DNA in maternal plasma correlates with gestational age. Fetal DNA fraction was estimated by three different ways: 1. From the additional amount of chromosomes 13, 18, and 21 sequences for T13, T18, and T21 cases respectively. 2. From the depletion in amount of chromosome X sequences for male cases. 3. From the amount of chromosome Y sequences present for male cases. The horizontal dashed line represents the estimated minimum fetal DNA fraction required for the detection of aneuploidy. For each sample, the values of fetal DNA fraction calculated from the data of different chromosomes were averaged. There is a statistically significant correlation between the average fetal DNA fraction and gestational age ($p=0.0051$). The dashed line represents the simple linear regression line between the average fetal DNA fraction and gestational age. The R2 value represents the square of the correlation coefficient. FIG. **2** suggests that the present method may be employed at a very early stage of pregnancy. The data were obtained from the 10-week stage and later because that is the earliest stage at which chorionic villi sampling is done. (Amniocentesis is done later). From the level of the confidence interval, one would expect to obtain meaningful data as early as 4 weeks gestational age, or possibly earlier.

FIG. **3** is a histogram showing size distribution of maternal and fetal DNA in maternal plasma. It shows the size distribution of total and chromosome Y specific fragments obtained from 454 sequencing of maternal plasma DNA from a normal male pregnancy. The distribution is normalized to sum to 1. The numbers of total reads and reads mapped to the Y-chromosome are 144992 and 178 respectively. Inset: Cumulative fetal DNA fraction as a function of sequenced fragment size. The error bars correspond to the standard error of the fraction estimated assuming the error of the counts of sequenced fragments follow Poisson statistics.

FIG. **4** is a pair of line graphs showing distribution of sequence tags around transcription start sites (TSS) of ReSeq genes on all autosomes and chromosome X from plasma DNA sample of a normal male pregnancy (top, FIG. **4**A) and randomly sheared genomic DNA control (bottom, FIG. **4**B). The number of tags within each 5 bp window was counted within ±1000 bp region around each TSS, taking into account the strand each sequence tag mapped to. The counts from all transcription start sites for each 5 bp window were summed and normalized to the median count among the 400 windows. A moving average was used to smooth the data. A peak in the sense strand represents the beginning of a nucleosome, while a peak in the anti-sense strand represents the end of a nucleosome. In the plasma DNA sample shown here, five well-positioned nucleosomes are observed downstream of transcription start sites and are represented as grey ovals. The number below within each oval represents the distance in base pairs between adjacent peaks in the sense and anti-sense strands, corresponding to the size of the inferred nucleosome. No obvious pattern is observed for the genomic DNA control.

FIG. **5**A is a scatter plot graph showing the mean sequence tag density for each chromosome of all samples, including cell-free plasma DNA from pregnant women and male donor, as well as genomic DNA control from male donor, is plotted above. Exceptions are chromosomes 13, 18 and 21, where cell-free DNA samples from women carrying aneuploid fetuses are excluded. The error bars represent standard deviation. The chromosomes are ordered by their G/C content. G/C content of each chromosome relative to the genome-wide value (41%) is also plotted. FIG. **5**B is a scatter plot of mean sequence tag density for each chromosome versus G/C content of the chromosome. The correlation coefficient is 0.927, and the correlation is statistically significant ($p<10^{-9}$).

FIG. **5**C is a scatter plot of the standard deviation of sequence tag density of each chromosome versus G/C content

of the chromosome. The correlation coefficient between standard deviation of sequence tag density and the absolute deviation of chromosomal G/C content from the genome-wide G/C content is 0.963, and the correlation is statistically significant ($p<10-12$).

FIG. **6** is a scatter plot graph showing percent difference of chromosome X sequence tag density of all samples as compared to the median chromosome X sequence tag density of all female pregnancies. All male pregnancies show underrepresentation of chromosome X.

FIG. **7** is a scatter plot graph showing a comparison of the estimation of fetal DNA fraction for cell-free DNA samples from 12 male pregnancies using sequencing data from chromosomes X and Y. The dashed line represents a simple linear regression line, with a slope of 0.85. The R2 value represents the square of the correlation coefficient. There is a statistically significant correlation between fetal DNA fraction estimated from chromosomes X and Y ($p=0.0015$).

FIG. **8** is a line graph showing length distribution of sequenced fragments from maternal cell-free plasma DNA sample of a normal male pregnancy at 1bp resolution. Sequencing was done on the 454/Roche platform. Reads that have at least 90% mapping to the human genome with greater than or equal to 90% accuracy are retained, totaling 144992 reads. Y-axis represents the number of reads obtained. The median length is 177 bp while the mean length is 180 bp.

FIG. **9** is a schematic illustrating how sequence tag distribution is used to detect the over and under-representation of any chromosome, i.e., a trisomy (over representation) or a missing chromosome (typically an X or Y chromosome, since missing autosomes are generally lethal). As shown in left panels A and C, one first plots the number of reads obtained versus a window that is mapped to a chromosome coordinate that represents the position of the read along the chromosome. That is, chromosome 1 (panel A) can be seen to have about 2.8×108 bp. It would have this number divided by 50 kb windows. These values are replotted (panels B and D) to show the distribution of the number of sequence tags/50 kb window. The term "bin" is equivalent to a window. From this analysis, one can determine a median number of reads M for each chromosome, which, for purposes of illustration, may be observed along the x axis at the approximate center of the distribution and may be said to be higher if there are more sequence tags attributable to that chromosome. For chromosome 1, illustrated in panels A and B, one obtains a median M1. By taking the median M of all 22 autosomes, one obtains a normalization constant N that can be used to correct for differences in sequences obtained in different runs, as can be seen in Table 1. Thus, the normalized sequence tag density for chromosome 1 would be M1/N; for chromosome 22 it would be M22/N. Close examination of panel A, for example would show that towards the zero end of the chromosome, this procedure obtained about 175 reads per 50 kb window. In the middle, near the centromere, there were no reads, because this portion of the chromosome is ill defined in the human genome library.

That is, in the left panels (A and C), one plots the distribution of reads per chromosome coordinate, i.e., chromosomal position in terms of number of reads within each 50 kb nonoverlapping sliding window. Then, one determines the distribution of the number of sequence tags for each 50 kb window, and obtains a median number of sequence tags per chromosome for all autosomes and chromosome X (Examples of chr 1 [top] and chr 22 [bottom] are illustrated here). These results are referred to as M. The median of the 22 values of M (from all autosomes, chromosomes 1 through 22) is used as the normalization constant N. The normalized sequence tag density of each chromosome is M/N (e.g., chr 1: M1/N; chr 22: M22/N). Such normalization is necessary to compare different patient samples since the total number of sequence tags (thus, the sequence tag density) for each patient sample is different (the total number of sequence tags fluctuates between ~8 to ~12 million). The analysis thus flows from frequency of reads per coordinate (A and C) to # reads per window (B and D) to a combination of all chromosomes.

FIG. **10** is a scatter plot graph showing data from different samples, as in FIG. **1**, except that bias for G/C sampling has been eliminated.

FIG. **11** is a scatter plot graph showing the weight given to different sequence samples according to percentage of G/C content, with lower weight given to samples with a higher G/C content. G/C content ranges from about 30% to about 70%; weight can range over a factor of about 3.

FIG. **12** is a scatter plot graph which illustrates results of selected patients as indicated on the x axis, and, for each patient, a distribution of chromosome representation on the Y axis, as deviating from a representative t statistic, indicated as zero.

FIG. **13** is a scatter plot graph showing the minimum fetal DNA percentage of which over- or under-representation of a chromosome could be detected with a 99.9% confidence level for chromosomes 21, 18, 13 and Chr. X, and a value for all other chromosomes.

FIG. **14** is a scatter plot graph showing a linear relationship between log 10 of minimum fetal DNA percentage that is needed versus log 10 of the number of reads required.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

Overview

Definitions

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by those of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are described. Generally, nomenclatures utilized in connection with, and techniques of, cell and molecular biology and chemistry are those well known and commonly used in the art. Certain experimental techniques, not specifically defined, are generally performed according to conventional methods well known in the art and as described in various general and more specific references that are cited and discussed throughout the present specification. For purposes of the clarity, following terms are defined below.

"Sequence tag density" means the normalized value of sequence tags for a defined window of a sequence on a chromosome (in a preferred embodiment the window is about 50 kb), where the sequence tag density is used for comparing different samples and for subsequent analysis. A "sequence tag" is a DNA sequence of sufficient length that it may be assigned specifically to one of chromosomes 1-22, X or Y. It does not necessarily need to be, but may be non-repetitive within a single chromosome. A certain, small degree of mismatch (0-1) may be allowed to account for minor polymorphisms that may exist between the reference genome and the individual genomes (maternal and fetal) being mapped. The value of the sequence tag density is normalized within a sample. This can be done by counting the number of tags falling within each window on a chromosome; obtaining a

median value of the total sequence tag count for each chromosome; obtaining a median value of all of the autosomal values; and using this value as a normalization constant to account for the differences in total number of sequence tags obtained for different samples. A sequence tag density as calculated in this way would ideally be about 1 for a disomic chromosome. As further described below, sequence tag densities can vary according to sequencing artifacts, most notably G/C bias; this is corrected as described. This method does not require the use of an external standard, but, rather, provides an internal reference, derived from al of the sequence tags (genomic sequences), which may be, for example, a single chromosome or a calculated value from all autosomes.

"T21" means trisomy 21.

"T18" means trisomy 18.

"T13" means trisomy 13.

"Aneuploidy" is used in a general sense to mean the presence or absence of an entire chromosome, as well as the presence of partial chromosomal duplications or deletions or kilobase or greater size, as opposed to genetic mutations or polymorphisms where sequence differences exist.

"Massively parallel sequencing" means techniques for sequencing millions of fragments of nucleic acids, e.g., using attachment of randomly fragmented genomic DNA to a planar, optically transparent surface and solid phase amplification to create a high density sequencing flow cell with millions of clusters, each containing ~1,000 copies of template per sq. cm. These templates are sequenced using four-color DNA sequencing-by-synthesis technology. See, products offered by Illumina, Inc., San Diego, Calif. In the present work, sequences were obtained, as described below, with an Illumina/Solexa 1G Genome Analyzer. The Solexa/Illumina method referred to below relies on the attachment of randomly fragmented genomic DNA to a planar, optically transparent surface. In the present case, the plasma DNA does not need to be sheared. Attached DNA fragments are extended and bridge amplified to create an ultra-high density sequencing flow cell with ≧50 million clusters, each containing ~1,000 copies of the same template. These templates are sequenced using a robust four-color DNA sequencing-by-synthesis technology that employs reversible terminators with removable fluorescent dyes. This novel approach ensures high accuracy and true base-by-base sequencing, eliminating sequence-context specific errors and enabling sequencing through homopolymers and repetitive sequences.

High-sensitivity fluorescence detection is achieved using laser excitation and total internal reflection optics. Short sequence reads are aligned against a reference genome and genetic differences are called using specially developed data analysis pipeline software.

Copies of the protocol for whole genome sequencing using Solexa technology may be found at BioTechniques® Protocol Guide 2007 Published December 2006: p 29, www(dot) biotechniques.com/default.asp?
page=protocol&subsection=article_display&id=112378.
Solexa's oligonucleotide adapters are ligated onto the fragments, yielding a fully-representative genomic library of DNA templates without cloning. Single molecule clonal amplification involves six steps: Template hybridization, template amplification, linearization, blocking 3' ends, denaturation and primer hybridization. Solexa's Sequencing-by-Synthesis utilizes four proprietary nucleotides possessing reversible fluorophore and termination properties. Each sequencing cycle occurs in the presence of all four nucleotides.

The presently used sequencing is preferably carried out without a preamplification or cloning step, but may be combined with amplification-based methods in a microfluidic chip having reaction chambers for both PCR and microscopic template-based sequencing. Only about 30 bp of random sequence information are needed to identify a sequence as belonging to a specific human chromosome. Longer sequences can uniquely identify more particular targets. In the present case, a large number of 25 bp reads were obtained, and due to the large number of reads obtained, the 50% specificity enabled sufficient sequence tag representation.

Further description of a massively parallel sequencing method, which employed the below referenced 454 method is found in Rogers and Ventner, "Genomics: Massively parallel sequencing," *Nature,* 437, 326-327 (15 Sep. 2005). As described there, Rothberg and colleagues (Margulies, M. et al. *Nature* 437, 376-380 (2005)), have developed a highly parallel system capable of sequencing 25 million bases in a four-hour period—about 100 times faster than the current state-of-the-art Sanger sequencing and capillary-based electrophoresis platform. The method could potentially allow one individual to prepare and sequence an entire genome in a few days. The complexity of the system lies primarily in the sample preparation and in the microfabricated, massively parallel platform, which contains 1.6 million picoliter-sized reactors in a 6.4-cm^2 slide. Sample preparation starts with fragmentation of the genomic DNA, followed by the attachment of adaptor sequences to the ends of the DNA pieces. The adaptors allow the DNA fragments to bind to tiny beads (around 28μ in diameter). This is done under conditions that allow only one piece of DNA to bind to each bead. The beads are encased in droplets of oil that contain all of the reactants needed to amplify the DNA using a standard tool called the polymerase chain reaction. The oil droplets form part of an emulsion so that each bead is kept apart from its neighbor, ensuring the amplification is uncontaminated. Each bead ends up with roughly 10 million copies of its initial DNA fragment. To perform the sequencing reaction, the DNA-template-carrying beads are loaded into the picoliter reactor wells—each well having space for just one bead. The technique uses a sequencing-by-synthesis method developed by Uhlen and colleagues, in which DNA complementary to each template strand is synthesized. The nucleotide bases used for sequencing release a chemical group as the base forms a bond with the growing DNA chain, and this group drives a light-emitting reaction in the presence of specific enzymes and luciferin. Sequential washes of each of the four possible nucleotides are run over the plate, and a detector senses which of the wells emit light with each wash to determine the sequence of the growing strand. This method has been adopted commercially by 454 Life Sciences.

Further examples of massively parallel sequencing are given in US 20070224613 by Strathmann, published Sep. 27, 2007, entitled "Massively Multiplexed Sequencing." Also, for a further description of massively parallel sequencing, see US 2003/0022207 to Balasubramanian, et al., published Jan. 30, 2003, entitled "Arrayed polynucleotides and their use in genome analysis."

General Description of Method and Materials

Overview

Non-invasive prenatal diagnosis of aneuploidy has been a challenging problem because fetal DNA constitutes a small percentage of total DNA in maternal blood (13) and intact fetal cells are even rarer (6, 7, 9, 31, 32). We showed in this study the successful development of a truly universal, polymorphism-independent non-invasive test for fetal aneuploidy. By directly sequencing maternal plasma DNA, we could detect fetal trisomy 21 as early as 14th week of gestation. Using cell-free DNA instead of intact cells allows one to

avoid complexities associated with microchimerism and foreign cells that might have colonized the mother; these cells occur at such low numbers that their contribution to the cell-free DNA is negligible (33, 34). Furthermore, there is evidence that cell-free fetal DNA clears from the blood to undetectable levels within a few hours of delivery and therefore is not carried forward from one pregnancy to the next (35-37).

Rare forms of aneuploidy caused by unbalanced translocations and partial duplication of a chromosome are in principle detectable by the approach of shotgun sequencing, since the density of sequence tags in the triplicated region of the chromosome would be higher than the rest of the chromosome. Detecting incomplete aneuploidy caused by mosaicism is also possible in principle but may be more challenging, since it depends not only on the concentration of fetal DNA in maternal plasma but also the degree of fetal mosaicism. Further studies are required to determine the effectiveness of shotgun sequencing in detecting these rare forms of aneuploidy.

The present method is applicable to large chromosomal deletions, such as 5p-Syndrome (five p minus), also known as Cat Cry Syndrome or Cri du Chat Syndrome. 5p-Syndrome is characterized at birth by a high-pitched cry, low birth weight, poor muscle tone, microcephaly, and potential medical complications. Similarly amenable disorders addressed by the present methods are p-, monosomy 9P, otherwise known as Alfi's Syndrome or 9P-, 22q11.2 deletion syndrome, Emanuel Syndrome, also known in the medical literature as the Supernumerary Der(22) Syndrome, trisomy 22, Unbalanced 11/22 Translocation or partial trisomy 11/22, Microdeletion and Microduplication at 16p11.2, which is associated with autism, and other deletions or imbalances, including those that are presently unknown.

An advantage of using direct sequencing to measure aneuploidy non-invasively is that it is able to make full use of the sample, while PCR based methods analyze only a few targeted sequences. In this study, we obtained on average 5 million reads per sample in a single run, of which ~66,000 mapped to chromosome 21. Since those 5 million reads represent only a portion of one human genome, in principle less than one genomic equivalent of DNA is sufficient for the detection of aneuploidy using direct sequencing. In practice, a larger amount of DNA was used since there is sample loss during sequencing library preparation, but it may be possible to further reduce the amount of blood required for analysis.

Mapping shotgun sequence information (i.e., sequence information from a fragment whose physical genomic position is unknown) can be done in a number of ways, which involve alignment of the obtained sequence with a matching sequence in a reference genome. See, Li et al., "Mapping short DNA sequencing reads and calling variants using mapping quality score," *Genome Res.*, 2008 Aug. 19. [Epub ahead of print].

We observed that certain chromosomes have large variations in the counts of sequenced fragments (from sample to sample, and that this depends strongly on the G/C content (FIG. **1**A) It is unclear at this point whether this stems from PCR artifacts during sequencing library preparation or cluster generation, the sequencing process itself, or whether it is a true biological effect relating to chromatin structure. We strongly suspect that it is an artifact since we also observe G/C bias on genomic DNA control, and such bias on the Solexa sequencing platform has recently been reported (38, 39). It has a practical consequence since the sensitivity to aneuploidy detection will vary from chromosome to chromosome; fortunately the most common human aneuploidies (such as 13, 18, and 21) have low variation and therefore high detection sensitivity. Both this problem and the sample volume limitations may possibly be resolved by the use of single molecule sequencing technologies, which do not require the use of PCR for library preparation (40).

Plasma DNA samples used in this study were obtained about 15 to 30 minutes after amniocentesis or chorionic villus sampling. Since these invasive procedures disrupt the interface between the placenta and maternal circulation, there have been discussions whether the amount of fetal DNA in maternal blood might increase following invasive procedures. Neither of the studies to date have observed a significant effect (41, 42).

Our results support this conclusion, since using the digital PCR assay we estimated that fetal DNA constituted less than or equal to 10% of total cell-free DNA in the majority of our maternal plasma samples. This is within the range of previously reported values in maternal plasma samples obtained prior to invasive procedures (13). It would be valuable to have a direct measurement addressing this point in a future study.

The average fetal DNA fraction estimated from sequencing data is higher than the values estimated from digital PCR data by an average factor of two ($p<0.005$, paired t-test on all male pregnancies that have complete set of data). One possible explanation for this is that the PCR step during Solexa library preparation preferentially amplifies shorter fragments, which others have found to be enriched for fetal DNA (22, 23). Our own measurements of length distribution on one sample do not support this explanation, but nor can we reject it at this point. It should also be pointed out that using the sequence tags we find some variation of fetal fraction even in the same sample depending on which chromosome we use to make the calculation (FIG. **7**, Table 1). This is most likely due to artifacts and errors in the sequencing and mapping processes, which are substantial—recall that only half of the sequence tags map to the human genome with one error or less. Finally, it is also possible that the PCR measurements are biased since they are only sampling a tiny fraction of the fetal genome.

Our sequencing data suggest that the majority of cell-free plasma DNA is of apoptotic origin and shares features of nucleosomal DNA. Since nucleosome occupancy throughout the eukaryotic genome is not necessarily uniform and depends on factors such as function, expression, or sequence of the region (30, 43), the representation of sequences from different loci in cell-free maternal plasma may not be equal, as one usually expects in genomic DNA extracted from intact cells. Thus, the quantity of a particular locus may not be representative of the quantity of the entire chromosome and care must be taken when one designs assays for measuring gene dosage in cell-free maternal plasma DNA that target only a few loci.

Historically, due to risks associated with chorionic villus sampling and amniocentesis, invasive diagnosis of fetal aneuploidy was primarily offered to women who were considered at risk of carrying an aneuploid fetus based on evaluation of risk factors such as maternal age, levels of serum markers, and ultrasonographic findings. Recently, an American College of Obstetricians and Gynecologists (ACOG) Practice Bulletin recommended that "invasive diagnostic testing for aneuploidy should be available to all women, regardless of maternal age" and that "pretest counseling should include a discussion of the risks and benefits of invasive testing compared with screening tests" (2).

A noninvasive genetic test based on the results described here and in future large-scale studies would presumably carry the best of both worlds: minimal risk to the fetus while providing true genetic information. The costs of the assay are already fairly low; the sequencing cost per sample as of this

writing is about $700 and the cost of sequencing is expected to continue to drop dramatically in the near future.

Shotgun sequencing can potentially reveal many more previously unknown features of cell-free nucleic acids such as plasma mRNA distributions, as well as epigenetic features of plasma DNA such as DNA methylation and histone modification, in fields including perinatology, oncology and transplantation, thereby improving our understanding of the basic biology of pregnancy, early human development and disease.

Sequencing Methods

Commercially available sequencing equipment was used in the present illustrative examples, namely the Solexa/Illumina sequencing platform and the 454/Roche platform. It will be apparent to those skilled in the art that a number of different sequencing methods and variations can be used. One sequencing method that can be used to advantage in the present methods involves paired end sequencing. Fluorescently labeled sequencing primers could be used to simultaneously sequence both strands of a dsDNA template, as described e.g., in Wiemann et al. (*Anal. Biochem.* 224: 117 [1995]; *Anal. Biochem.* 234: 166 [1996]. Recent examples of this technique have demonstrated multiplex co-sequencing using the four-color dye terminator reaction chemistry pioneered by Prober et al. (*Science* 238: 336 [1987]). Solexa/Illumina offers a "Paired End Module" to its Genome Analyzer. Using this module, after the Genome Analyzer has completed the first sequencing read, the Paired-End Module directs the resynthesis of the original templates and the second round of cluster generation. The Paired-End Module is connected to the Genome Analyzer through a single fluidic connection. In addition, 454 has developed a protocol to generate a library of Paired End reads. These Paired End reads are approximately 84-nucleotide DNA fragments that have a 44-mer adaptor sequence in the middle flanked by a 20-mer sequence on each side. The two flanking 20-mers are segments of DNA that were originally located approximately 2.5 kb apart in the genome of interest.

By using paired end reads in the present method, one may obtain more sequence information from a given plasma DNA fragment, and, significantly, one may also obtain sequence information from both ends of the fragment. The fragment is mapped to the human genome as explained here elsewhere. After mapping both ends, one may deduce the length of the starting fragment. Since fetal DNA is known to be shorter than maternal DNA fragments circulating in plasma, one may use this information about the length of the DNA fragment to effectively increase the weight given to sequences obtained from shorter (e.g., about 300 bp or less) DNA fragments. Methods for weighting are given below.

Another method for increasing sensitivity to fetal DNA is to focus on certain regions within the human genome. One may use sequencing methods which select a priori sequences which map to the chromosomes of interest (as described here elsewhere, such as 18, 21, 13, X and Y). One may also choose to focus, using this method, on partial chromosomal deletions, such as 22q11 deletion syndrome. Other microdeletions and microduplications are set forth in Table 1 of US 2005/0181410, published Aug. 18, 2005 under the title "Methods and apparatuses for achieving precision genetic diagnosis."

In sequencing selected subsequences, one may employ sequence-based methodologies such as sequencing by array, or capture beads with specific genomic sequences used as capture probes. The use of a sequencing array can be implemented as described in Chetverin et al., "Oligonucleotide arrays: new concepts and possibilities," *Biotechnology* (NY). 1994 November; 12(11):1093-9, as well as Rothberg, US 2002/0012930 A1 entitled "Method of Sequencing a Nucleic Acid," and Reeve et al., "Sequencing by Hybridization," U.S. Pat. No. 6,399,364. In these methods, the target nucleic acid to be sequenced may be genomic DNA, cDNA or RNA. The sample is rendered single stranded and captured under hybridizing conditions with a number of single stranded probes which are catalogued by bar coding or by physical separation in an array. Emulsion PCR, as used in the 454 system, the SOLiD system, and Polonator (Dover Systems) and others may also be used, where capture is directed to specific target sequences, e.g., genome sequences mapping uniquely to chromosome 21 or other chromosome of interest, or to a chromosome region such as 15q11 (Prader-Willi syndrome), or excessive CGG repeats in the FMR1 gene (fragile X syndrome).

The subsequencing method is in one aspect contrary to conventional massively parallel sequencing methodologies, which seek to obtain all of the sequence information in a sample. This alternative method selectively ignores certain sequence information by using a sequencing method which selectively captures sample molecules containing certain predefined sequences. One may also use the sequencing steps exactly as exemplified, but in mapping the sequence fragments obtained, give greater weight to sequences which map to areas known to be more reliable in their coverage, such as exons. Otherwise, the method proceeds as described below, where one obtains a large number of sequence reads from one or more reference chromosomes, which are compared to a large number of reads obtained from a chromosome of interest, after accounting for variations arising from chromosomal length, G/C content, repeat sequences and the like.

One may also focus on certain regions within the human genome according to the present methods in order to identify partial monosomies and partial trisomies. As described below, the present methods involve analyzing sequence data in a defined chromosomal sliding "window," such as contiguous, nonoverlapping 50 Kb regions spread across a chromosome. Partial trisomies of 13q, 8p (8p23.1), 7q, distal 6p, 5p, 1q (3q25.1), 2q, 1q (1q42.1 and 1q21-qter), partial Xpand monosomy 4q35.1 have been reported, among others. For example, partial duplications of the long arm of chromosome 18 can result in Edwards syndrome in the case of a duplication of 18q21.1-qter (See, Mewar et al., "Clinical and molecular evaluation of four patients with partial duplications of the long arm of chromosome 18," *Am J Hum Genet.* 1993 December; 53(6):1269-78).

Shotgun Sequencing of Cell-Free Plasma DNA

Cell-free plasma DNA from 18 pregnant women and a male donor, as well as whole blood genomic DNA from the same male donor, were sequenced on the Solexa/Illumina platform. We obtained on average ~10 million 25 bp sequence tags per sample. About 50% (i.e., ~5 million) of the reads mapped uniquely to the human genome with at most 1 mismatch against the human genome, covering ~4% of the entire genome. An average of ~154,000, ~135,000, ~66,000 sequence tags mapped to chromosomes 13, 18, and 21, respectively. The number of sequence tags for each sample is detailed in the following Table 1 and Table 2.

TABLE 1

Sample	Fetal Karyotype	Gestational Age (weeks)	Volume of Plasma	Amount of DNA	Approximate Amount of Input DNA *	Total Number of Sequence Tags
P1 Plasma DNA§	47XX + 21	35	1.6	761	8.0	8206694
P2 Plasma DNA§	47XY + 21	18	1.4	585	5.2	7751384
P6 Plasma DNA§	47XX + 21	14	1.6	410	4.3	6699183
P7 Plasma DNA§	47XY + 21	18	2.2	266	3.8	8324473
P14 Plasma DNA§	47XX + 21	23	3.2	57	1.2	8924944
P17 Plasma DNA§	47XX + 21	16	2.3	210	3.2	11599833
P19 Plasma DNA§	46XY	18	3.2	333	7.0	7305417
P20 Plasma DNA§	47XY + 21	18	1.3	408	3.6	11454876
P23 Plasma DNA§	46XY	10	1.6	258	2.7	11851612
P26 Plasma DNA§	46XY	13	3.0	340	6.7	11471297
P31 Plasma DNA§	46XY	20	2.2	278	4.0	8967562
P40 Plasma DNA§	46XY	11	2.6	217	3.7	9205197
P42 Plasma DNA§	46XY	11	3.0	276	5.5	8364774
P52 Plasma DNA§	47XY + 21	25	1.6	645	6.8	9192596
P53 Plasma DNA§	47XX + 21	19	1.6	539	5.7	9771887
P57 Plasma DNA§	47XX + 18	23	2.0	199	2.6	15041417
P59 Plasma DNA§	47XY + 18	21	2.0	426	5.6	11910483
P64 Plasma DNA§	47XY + 13	17	1.8	204	2.4	12097478
Male Donor Plasma DNA§	—	—	1.8	485	5.8	6669125
Male Donor Whole Blood Genomic DNA§	—	—	—	—	2.1	8519495
P25 Plasma DNA¶	46XY	11	5.6	132	4.9	242599
P13 Plasma DNA§	46XY	18	5.6	77	2.9	4168455

TABLE 2

Sample	Number of Sequence Tags Mapped Uniquely to the Human Genome (hg18) with At Most 1 Mismatch	% Fetal DNA Estimated By Digital PCR with SRY Assay (male fetuses)	% Fetal DNA Estimated by ChrY Sequence Tags (male fetuses)	% Fetal DNA Estimated by Depletion of ChrX Sequence Tags (male fetuses)	% Fetal DNA Estimated by Addition of Trisomic Chromosome Sequence Tags (aneuploid fetuses)	Overall G/C content Of Sequence Tags (%)
P1 Plasma DNA§	4632637	—	—	—	35.0	43.65
P2 Plasma DNA§	4313884	6.4	15.4	21.6	15.5	48.72
P6 Plasma DNA§	3878383	—	—	—	22.9	44.78
P7 Plasma DNA§	4294865	9.1	31.0	33.8	28.6	48.07
P14 Plasma DNA§	3603767	—	—	—	30.5	46.38
P17 Plasma DNA§	5968932	—	—	—	7.8	44.29
P19 Plasma DNA§	3280521	<5.9‡	4.14	21.5	—	50.09
P20 Plasma DNA§	6032684	10.0	15.7	11.3	11.5	44.02
P23 Plasma DNA§	6642795	5.3	12.2	9.6	—	43.80
P26 Plasma DNA§	3851477	10.3	18.2	14.2	—	42.51
P31 Plasma DNA§	4683777	Missing data‡	13.2	17.0	—	48.27
P40 Plasma DNA§	4187561	8.6	20.0	17.1	—	42.65
P42 Plasma DNA§	4315527	<4.4‡	9.7	7.9	—	44.14
P52 Plasma DNA§	5126837	6.3	25.0	26.3	26.4	44.34
P53 Plasma DNA§	5434222	—	—	—	25.8	44.18
P57 Plasma DNA§	7470487	—	—	—	23.0	42.89
P59 Plasma DNA§	6684871	26.4	44.0	39.8	45.1	43.64
P64 Plasma DNA§	6701148	<4.4‡	14.0	8.9	16.7	44.21
Male Donor Plasma DNA§	3692931	—	—	—	—	48.30
Male Donor Whole Blood Genomic DNA§	5085412	—	—	—	—	46.53
P25 Plasma DNA¶	144992†	—	—	—	—	41.38
P13 Plasma DNA§	2835333	9.8	5.7	n/a‖	—	39.60

The volume of plasma is the volume used for Sequencing Library Creation (ml). The amount of DNA is in Plasma (cell equivalent/ml plasma)*. The approximate amount of input DNA is that use for Sequencing Library Construction (ng).

*As quantified by digital PCR with EIF2C1 Taqman Assay, converting from copies to ng assuming 6.6 pg/cell equivalent.

†For 454 sequencing, this number represents the number of reads with at least 90% accuracy and 90% coverage when mapped to hg18.

‡Insufficient materials were available for quantifying fetal DNA % with digital PCR for these samples (either no samples remained for analysis or there was insufficient sampling).

§Sequenced on Solexa/Illumina platform;

¶Sequenced on 454/Roche platform

‖Sample P13 was the first to be analyzed by shotgun sequencing. It was a normal fetus and the chromosome value was clearly disomic. However, there were some irregularities with this sample and it was not included in further analysis. This sample was sequenced on a different Solexa instrument than the rest of the samples of this study, and it was sequenced in the presence of a number of samples of unknown origin. The G/C content of this sample was lower than the G/C bias of the human genome, while the rest of the samples are above. It had the lowest number of reads, and also the smallest number of reads mapped successfully to the human genome. This sample appeared to be outlier in sequence tag density for most chromosomes and the fetal DNA fraction calculated from chromosomes X was not well defined. For these reasons we suspect that the irregularities are due to technical problems with the sequencing process.

In Table 1 and Table 2, each sample represents a different patient, e.g., P1 in the first row. The total number of sequence tags varied but was frequently was in the 10 million range, using the Solexa technology. The 454 technology used for P25 and P13 gave a lower number of reads.

We observed a non-uniform distribution of sequence tags across each chromosome. This pattern of intra-chromosomal variation was common among all samples, including randomly sheared genomic DNA, indicating the observed variation was most probably due to sequencing artifacts. We applied an arbitrary sliding window of 50 kb across each chromosome and counted the number of tags falling within each window. The window can be varied in size to account for larger numbers of reads (in which cases a smaller window, e.g., 10 kb, gives a more detailed picture of a chromosome) or a smaller number of reads, in which case a larger window (e.g., 100 kb) may still be used and will detect gross chromosome deletions, omissions or duplications. The median count per 50 kb window for each chromosome was selected. The median of the autosomal values (i.e., 22 chromosomes) was used as a normalization constant to account for the differences in total number of sequence tags obtained for different samples. The inter-chromosomal variation within each sample was also consistent among all samples (including genomic DNA control). The mean sequence tag density of each chromosome correlates with the G/C content of the chromosome ($p<10^{-9}$) (FIG. **5**A, **5**B). The standard deviation of sequence tag density for each chromosome also correlates with the absolute degree of deviation in chromosomal G/C content from the genome-wide G/C content ($p<10^{-12}$) (FIG. **5**A, **5**C). The G/C content of sequenced tags of all samples (including the genomic DNA control) was on average 10% higher than the value of the sequenced human genome (41%) (21) (Table 2), suggesting that there is a strong G/C bias stemming from the sequencing process. We plotted in FIG. **1**A the sequence tag density for each chromosome (ordered by increasing G/C content) relative to the corresponding value of the genomic DNA control to remove such bias.

Detection of Fetal Aneuploidy

The distribution of chromosome 21 sequence tag density for all 9 T21 pregnancies is clearly separated from that of pregnancies bearing disomy 21 fetuses ($p<10^{-5}$), Student's t-test) (FIGS. **1**A and **1**B). The coverage of chromosome 21 for T21 cases is about ~4-18% higher (average ~11%) than that of the disomy 21 cases. Because the sequence tag density of chromosome 21 for T21 cases should be $(1+\epsilon/2)$ of that of disomy 21 pregnancies, where ϵ is the fraction of total plasma DNA originating from the fetus, such increase in chromosome 21 coverage in T21 cases corresponds to a fetal DNA fraction of ~8%-35% (average ~23%) (Table 1, FIG. **2**). We constructed a 99% confidence interval of the distribution of chromosome 21 sequence tag density of disomy 21 pregnancies. The values for all 9 T21 cases lie outside the upper boundary of the confidence interval and those for all 9 disomy 21 cases lie below the boundary (FIG. **1**B). If we used the upper bound of the confidence interval as a threshold value for detecting T21, the minimum fraction of fetal DNA that would be detected is ~2%.

Plasma DNA of pregnant women carrying T18 fetuses (2 cases) and a T13 fetus (1 case) were also directly sequenced. Over-representation was observed for chromosome 18 and chromosome 13 in T18 and T13 cases respectively (FIG. **1**A). While there were not enough positive samples to measure a representative distribution, it is encouraging that all of these three positives are outliers from the distribution of disomy values. The T18 are large outliers and are clearly statistically significant ($p<10^{-7}$), while the statistical significance of the single T13 case is marginal ($p<0.05$). Fetal DNA fraction was also calculated from the over-represented chromosome as described above (FIG. **2**, Table 1).

Fetal DNA Fraction in Maternal Plasma

Using digital Taqman PCR for a single locus on chromosome 1, we estimated the average cell-free DNA concentration in the sequenced maternal plasma samples to be ~360 cell equivalent/ml of plasma (range: 57 to 761 cell equivalent/ml plasma) (Table 1), in rough accordance to previously reported values (13). The cohort included 12 male pregnancies (6 normal cases, 4 T21 cases, 1 T18 case and 1 T13 case) and 6 female pregnancies (5 T21 cases and 1 T18 case). DYS14, a multi-copy locus on chromosome Y, was detectable in maternal plasma by real-time PCR in all these pregnancies but not in any of the female pregnancies (data not shown). The fraction of fetal DNA in maternal cell-free plasma DNA is usually determined by comparing the amount of fetal specific locus (such as the SRY locus on chromosome Y in male pregnancies) to that of a locus on any autosome that is common to both the mother and the fetus using quantitative real-time PCR (13, 22, 23). We applied a similar duplex assay on a digital PCR platform (see Methods) to compare the counts of the SRY locus and a locus on chromosome 1 in male pregnancies. SRY locus was not detectable in any plasma DNA samples from female pregnancies. We found with digital PCR that for the majority samples, fetal DNA constituted §10% of total DNA in maternal plasma (Table 2), agreeing with previously reported values (13).

The percentage of fetal DNA among total cell-free DNA in maternal plasma can also be calculated from the density of sequence tags of the sex chromosomes for male pregnancies. By comparing the sequence tag density of chromosome Y of plasma DNA from male pregnancies to that of adult male plasma DNA, we estimated fetal DNA percentage to be on average ~19% (range: 4-44%) for all male pregnancies (Table 2, above, FIG. **2**). Because human males have 1 fewer chromosome X than human females, the sequence tag density of chromosome X in male pregnancies should be (1-e/2) of that of female pregnancies, where e is fetal DNA fraction. We indeed observed under-representation of chromosome X in male pregnancies as compared to that of female pregnancies (FIG. **5**). Based on the data from chromosome X, we estimated fetal DNA percentage to be on average ~19% (range: 8-40%) for all male pregnancies (Table 2, above, FIG. **2**). The fetal DNA percentage estimated from chromosomes X and Y for each male pregnancy sample correlated with each other ($p=0.0015$) (FIG. **7**).

We plotted in FIG. **2** the fetal DNA fraction calculated from the over-representation of trisomic chromosome in aneuploid pregnancies, and the under-representation of chromosome X and the presence of chromosome Y for male pregnancies against gestational age. The average fetal DNA fraction for each sample correlates with gestational age ($p=0.0051$), a trend that is also previously reported (13).

Size Distribution of Cell-Free Plasma DNA

We analyzed the sequencing libraries with a commercial lab-on-a-chip capillary electrophoresis system. There is a striking consistency in the peak fragment size, as well as the distribution around the peak, for all plasma DNA samples, including those from pregnant women and male donor. The peak fragment size was on average 261 bp (range: 256-264 bp). Subtracting the total length of the Solexa adaptors (92 bp) from 260 bp gives 169 bp as the actual peak fragment size. This size corresponds to the length of DNA wrapped in a chromatosome, which is a nucleosome bound to a H1 histone (24). Because the library preparation includes an 18-cycle PCR, there are concerns that the distribution might be biased.

To verify that the size distribution observed in the electropherograms is not an artifact of PCR, we also sequenced cell-free plasma DNA from a pregnant woman carrying a male fetus using the 454 platform. The sample preparation for this system uses emulsion PCR, which does not require competitive amplification of the sequencing libraries and creates product that is largely independent of the amplification efficiency. The size distribution of the reads mapped to unique locations of the human genome resembled those of the Solexa sequencing libraries, with a predominant peak at 176 bp, after subtracting the length of 454 universal adaptors (FIG. **3** and FIG. **8**). These findings suggest that the majority of cell-free DNA in the plasma is derived from apoptotic cells, in accordance with previous findings (22, 23, 25, 26).

Of particular interest is the size distribution of maternal and fetal DNA in maternal cell-free plasma. Two groups have previously shown that the majority of fetal DNA has size range of that of mono-nucleosome (<200-300 bp), while maternal DNA is longer. Because 454 sequencing has a targeted read-length of 250 bp, we interpreted the small peak at around 250 bp (FIG. **3** and FIG. **8**) as the instrumentation limit from sequencing higher molecular weight fragments. We plotted the distribution of all reads and those mapped to Y-chromosome (FIG. **3**). We observed a slight depletion of Y-chromosome reads in the higher end of the distribution. Reads <220 bp constitute 94% of Y-chromosome and 87% of the total reads. Our results are not in complete agreement with previous findings in that we do not see as dramatic an enrichment of fetal DNA at short lengths (22, 23). Future studies will be needed to resolve this point and to eliminate any potential residual bias in the 454 sample preparation process, but it is worth noting that the ability to sequence single plasma samples permits one to measure the distribution in length enrichments across many individual patients rather than measuring the average length enrichment of pooled patient samples.

Cell-Free Plasma DNA Shares Features of Nucleosomal DNA

Since our observations of the size distribution of cell-free plasma DNA suggested that plasma DNA is mainly apoptotic of origin, we investigated whether features of nucleosomal DNA and positioning are found in plasma DNA. One such feature is nucleosome positioning around transcription start sites. Experimental data from yeast and human have suggested that nucleosomes are depleted in promoters upstream of transcription start sites and nucleosomes are well-positioned near transcription start sites (27-30). We applied a 5 bp window spanning +/−1000 bp of transcription start sites of all RefSeq genes and counted the number of tags mapping to the sense and antisense strands within each window. A peak in the sense strand represents the beginning of a nucleosome while a peak in the antisense strand represents the end. After smoothing, we saw that for most plasma DNA samples, at least 3 well-positioned nucleosomes downstream of transcription start sites could be detected, and in some cases, up to 5 well-positioned nucleosomes could be detected, in rough accordance to the results of Schones et al. (27) (FIG. **4**). We applied the same analysis on sequence tags of randomly sheared genomic DNA and observed no obvious pattern in tag localization, although the density of tags was higher at the transcription start site (FIG. **4**).

Correction for Sequencing Bias

Shown in FIGS. **10** and **12** are results which may be obtained when sequence tag numbers are treated statistically based on data from the reference human genome. That is, for example, sequence tags from fragments with higher GC content may be overrepresented, and suggest an aneuploidy where none exists. The sequence tag information itself may not be informative, since only a small portion of the fragment ordinarily will be sequenced, while it is the overall G/C content of the fragment that causes the bias. Thus there is provided a method, described in detail in Examples 8 and 10, for correcting for this bias, and this method may facilitate analysis of samples which otherwise would not produce statistically significant results. This method, for correcting for G/C bias of sequence reads from massively parallel sequencing of a genome, comprises the step of dividing the genome into a number of windows within each chromosome and calculating the G/C content of each window. These windows need not be the same as the windows used for calculating sequence tag density; they may be on the order of 10 kb-30 kb in length, for example. One then calculates the relationship between sequence coverage and G/C content of each window by determining a number of reads per a given window and a G/C content of that window. The G/C content of each window is known from the human genome reference sequence. Certain windows will be ignored, i.e., with no reads or no G/C content. One then assigns a weight to the number of reads per a given window (i.e., the number of sequence tags assigned to that window) based on G/C content, where the weight has a relationship to G/C content such that increasing numbers of reads with increasing G/C content results in decreasing weight per increasing G/C content.

EXAMPLES

The examples below describe the direct sequencing of cell-free DNA from plasma of pregnant women with high throughput shotgun sequencing technology, obtaining on average 5 million sequence tags per patient sample. The sequences obtained were mapped to specific chromosomal locations. This enabled us to measure the over- and under-representation of chromosomes from an aneuploid fetus. The sequencing approach is polymorphism-independent and therefore universally applicable for the non-invasive detection of fetal aneuploidy. Using this method we successfully identified all 9 cases of trisomy 21 (Down syndrome), 2 cases of trisomy 18 and 1 case of trisomy 13 in a cohort of 18 normal and aneuploid pregnancies; trisomy was detected at gestational ages as early as the 14th week. Direct sequencing also allowed us to study the characteristics of cell-free plasma DNA, and we found evidence that this DNA is enriched for sequences from nucleosomes.

Example 1

Subject Enrollment

The study was approved by the Institutional Review Board of Stanford University. Pregnant women at risk for fetal aneuploidy were recruited at the Lucile Packard Children Hospital Perinatal Diagnostic Center of Stanford University during the period of April 2007 to May 2008. Informed consent was obtained from each participant prior to the blood draw. Blood was collected 15 to 30 minutes after amniocentesis or chorionic villus sampling except for 1 sample that was collected during the third trimester. Karyotype analysis was performed via amniocentesis or chorionic villus sampling to confirm fetal karyotype. 9 trisomy 21 (T21), 2 trisomy 18 (T18), 1 trisomy 13 (T13) and 6 normal singleton pregnancies were included in this study. The gestational age of the subjects at the time of blood draw ranged from 10 to 35 weeks (Table 1). Blood sample from a male donor was obtained from the Stanford Blood Center.

Example 2

Sample Processing and DNA Quantification

7 to 15 ml of peripheral blood drawn from each subject and donor was collected in EDTA tubes. Blood was centrifuged at 1600 g for 10 minutes. Plasma was transferred to microcentrifuge tubes and centrifuged at 16000 g for 10 minutes to remove residual cells. The two centrifugation steps were performed within 24 hours after blood collection. Cell-free plasma was stored at −80 C until further processing and was frozen and thawed only once before DNA extraction. DNA was extracted from cell-free plasma using QIAamp DNA Micro Kit (Qiagen) or NucleoSpin Plasma Kit (Macherey-Nagel) according to manufacturers' instructions. Genomic DNA was extracted from 2000 whole blood of the donors using QIAamp DNA Blood Mini Kit (Qiagen). Microfluidic digital PCR (Fluidigm) was used to quantify the amount of total and fetal DNA using Taqman assays targeting at the EIF2C1 locus on chromosome 1 (Forward: 5' GTTCGGCTTTCACCAGTCT 3' (SEQ ID NO: 1); Reverse: 5' CTCCATAGCTCTCCCCACTC 3' (SEQ ID NO: 2); Probe: 5' HEX-GCCCTGCCATGTGGAAGAT-BHQ1 3' (SEQ ID NO: 3); amplicon size: 81 bp) and the SRY locus on chromosome Y (Forward: 5' CGCTTAACATAGCAGAAGCA 3' (SEQ ID NO: 4); Reverse: 5' AGTTTCGAACTCTGGCACCT 3' (SEQ ID NO: 5); Probe: 5' FAM-TGTCGCACTCTCCTTGTTTTTGACA-BHQ1 3' (SEQ ID NO: 6); amplicon size: 84 bp) respectively. A Taqman assay targeting at DYS14 (Forward: 5' ATCGTCCATTTCCAGAATCA 3' (SEQ ID NO: 7); Reverse: 5' GTTGACAGCCGTGGAATC 3' (SEQ ID NO: 8); Probe: 5' FAM-TGCCACAGACTGAACTGAATGATTTTC-BHQ1 3' (SEQ ID NO: 9); amplicon size: 84 bp), a multi-copy locus on chromosome Y, was used for the initial determination of fetal sex from cell-free plasma DNA with traditional real-time PCR. PCR reactions were performed with 1×iQ Supermix (Bio-Rad), 0.1% Tween-20 (microfluidic digital PCR only), 300 nM primers, and 150 nM probes. The PCR thermal cycling protocol was 95 C for 10 min, followed by 40 cycles of 95 C for 15 s and 60 C for 1 min. Primers and probes were purchased form IDT.

Example 3

Sequencing

A total of 19 cell-free plasma DNA samples, including 18 from pregnant women and 1 from a male blood donor, and genomic DNA sample from whole blood of the same male donor, were sequenced on the Solexa/Illumina platform. ~1 to 8 ng of DNA fragments extracted from 1.3 to 5.6 ml cell-free plasma was used for sequencing library preparation (Table 1). Library preparation was carried out according to manufacturer's protocol with slight modifications. Because cell-free plasma DNA was fragmented in nature, no further fragmentation by nebulization or sonication was done on plasma DNA samples.

Genomic DNA from male donor's whole blood was sonicated (Misonix XL-2020) (24 cycles of 30 s sonication and 90 s pause), yielding fragments with size between 50 and 400 bp, with a peak at 150 bp. ~2 ng of the sonicated genomic DNA was used for library preparation. Briefly, DNA samples were blunt ended and ligated to universal adaptors. The amount of adaptors used for ligation was 500 times less than written on the manufacturer's protocol. 18 cycles of PCR were performed to enrich for fragments with adaptors using primers complementary to the adaptors. The size distributions of the sequencing libraries were analyzed with DNA 1000 Kit on the 2100 Bioanalyzer (Agilent) and quantified with microfluidic digital PCR (Fluidigm). The libraries were then sequenced using the Solexa 1G Genome Analyzer according to manufacturer's instructions.

Cell-free plasma DNA from a pregnant woman carrying a normal male fetus was also sequenced on the 454/Roche platform. Fragments of DNA extracted from 5.6 ml of cell-free plasma (equivalent to ~4.9 ng of DNA) were used for sequencing library preparation. The sequencing library was prepared according to manufacturer's protocol, except that no nebulization was performed on the sample and quantification was done with microfluidic digital PCR instead of capillary electrophoresis. The library was then sequenced on the 454 Genome Sequencer FLX System according to manufacturer's instructions.

Electropherograms of Solexa sequencing libraries were prepared from cell-free plasma DNA obtained from 18 pregnant women and 1 male donor. Solexa library prepared from sonicated whole blood genomic DNA from the male donor was also examined. For libraries prepared from cell-free DNA, all had peaks at average 261 bp (range: 256-264 bp). The actual peak size of DNA fragments in plasma DNA is ~168 bp (after removal of Solexa universal adaptor (92 bp)). This corresponds to the size of a chromatosome.

Example 4

Data Analysis

Shotgun Sequence Analysis

Solexa sequencing produced 36 to 50 bp reads. The first 25 bp of each read was mapped to the human genome build 36 (hg18) using ELAND from the Solexa data analysis pipeline. The reads that were uniquely mapped to the human genome having at most 1 mismatch were retained for analysis. To compare the coverage of the different chromosomes, a sliding window of 50 kb was applied across each chromosome, except in regions of assembly gaps and microsatellites, and the number of sequence tags falling within each window was counted and the median value was chosen to be the representative of the chromosome. Because the total number of sequence tags for each sample was different, for each sample, we normalized the sequence tag density of each chromosome (except chromosome Y) to the median sequence tag density among autosomes. The normalized values were used for comparison among samples in subsequent analysis. We estimated fetal DNA fraction from chromosome 21 for T21 cases, chromosome 18 from T18 cases, chromosome 13 from T13 case, and chromosomes X and Y for male pregnancies. For chromosome 21, 18, and 13, fetal DNA fraction was estimated as $2*(x-1)$, where x was the ratio of the over-represented chromosome sequence tag density of each trisomy case to the median chromosome sequence tag density of the all disomy cases. For chromosome X, fetal DNA was estimated as $2*(1-x)$, where x was the ratio of chromosome X sequence tag density of each male pregnancy to the median chromosome X sequence tag density of all female pregnancies. For chromosome Y, fetal DNA fraction was estimated as the ratio of chromosome Y sequence tag density of each male pregnancy to that of male donor plasma DNA. Because a small number of chromosome Y sequences were detected in female pregnancies, we only considered sequence tags falling within transcribed regions on chromosome Y and subtracted the median number of tags in female pregnancies from all samples; this amounted to a correction of a few percent. The width of 99% confidence intervals was calculated for all

disomy 21 pregnancies as t*s/vN, where N is the number of disomy 21 pregnancies, t is the t-statistic corresponding to a=0.005 with degree of freedom equals N−1, and s is the standard deviation. A confidence interval gives an estimated range of values, which is likely to include an unknown population parameter, the estimated range being calculated from a given set of sample data. (Definition taken from Valerie J. Easton and John H. McColl's Statistics Glossary v1.1)

To investigate the distribution of sequence tags around transcription start sites, a sliding window of 5 bp was applied from −1000 bp to +1000 bp of transcription start sites of all RefSeq genes on all chromosomes except chromosome Y. The number of sequence tags mapped to the sense and antisense strands within each window was counted. Moving average with a window of 10 data points was used to smooth the data. All analyses were done with Matlab.

We selected the sequence tags that mapped uniquely to the human genome with at most 1 mismatch (on average ~5 million) for analysis. The distribution of reads across each chromosome was examined. Because the distribution of sequence tags across each chromosome was non-uniform (possibly technical artifacts), we divided the length of each chromosome into non-overlapping sliding window with a fixed width (in this particular analysis, a 50 kbp window was used), skipping regions of genome assembly gaps and regions with known microsatellite repeats. The width of the window is should be large enough such that there are a sufficient number of sequence tags in each window, and should be small enough such that there are sufficient number of windows to form a distribution. With increasing sequencing depth (i.e., increasing total number of sequence tags), the window width can be reduced. The number of sequence tags in each window was counted. The distribution of the number of sequence tags per 50 kb for each chromosome was examined. The median value of the number of sequence tags per 50 kb (or 'sequence tag density') for each chromosome was chosen in order to suppress the effects of any under- or over-represented regions within the chromosome. Because the total number of sequence tags obtained for each sample was different, in order to compare among samples, we normalized each chromosomal sequence tag density value (except chromosome Y) by the median sequence tag density among all autosomes (non-sex chromosomes).

For the 454/Roche data, reads were aligned to the human genome build 36 (hg18, see hyper text transfer protocol (http) genome.ucsc.edu/cgi-bin/hgGateway) using the 454 Reference Mapper. Reads having accuracy of greater than or equal to 90% and coverage (i.e., fraction of read mapped) greater than or equal to 90% were retained for analysis. To study the size distribution of total and fetal DNA, the number of retained reads falling within each 10 bp window between 50 bp to 330 bp was counted. The number of reads falling within different size ranges may be studied, i.e., reads of between 50-60 bp, 60-70 bp, 70-80 bp, etc., up to about 320-330 bp, which is around the maximum read length obtained.

Example 5

Genome Data Retrieval

Information regarding G/C content, location of transcription start sites of RefSeq genes, location of assembly gaps and microsatellites were obtained from the UCSC Genome Browser.

Example 6

Nucleosome Enrichment

The distribution of sequence tags around transcription start sites (TSS) of RefSeq genes were analyzed (data not shown). The plots were similar to FIG. **4**. Each plot represented the distribution for each plasma DNA or gDNA sample. Data are obtained from three different sequencing runs (P1, P6, P52, P53, P26, P40, P42 were sequenced together; male genomic DNA, male plasma DNA, P2, P7, P14, P19, P31 were sequenced together; P17, P20, P23, P57, P59, P64 were sequenced together). The second batch of samples suffers greater G/C bias as observed from inter- and intra-chromosomal variation. Their distributions around TSS have similar trends with more tags at the TSS. Such trend is not as prominent as in the distributions of samples sequenced in other runs. Nonetheless, at least 3 well-positioned nucleosomes were detectable downstream of transcription start sites for most plasma DNA samples, suggesting that cell-free plasma DNA shares features of nucleosomal DNA, a piece of evidence that this DNA is of apoptotic origin.

Example 7

Calculating Fetal DNA Fraction in Maternal Plasma of Male Pregnancies

i. With Digital PCR Taqman Assays

Digital PCR is the amplification of single DNA molecule. DNA sample is diluted and distributed across multiple compartments such that on average there is less than 1 copy of DNA per compartment. A compartment displaying fluorescence at the end of a PCR represents the presence of at least one DNA molecule.

Assay for Total DNA: EIF2C1 (Chromosome 1)

Assay for Fetal DNA: SRY (Chromosome Y)

The count of positive compartments from the microfluidic digital PCR chip of each assay is converted to the most probable count according to the method described in the supporting information of the following reference: Warren L, Bryder D, Weissman I L, Quake S R (2006) Transcription factor profiling in individual hematopoietic progenitors by digital RT-PCR. Proc Nat Acad Sci, 103: 17807-12.

Fetal DNA Fraction ϵ=(SRY count)/(EIF2C1 count/2)

ii. With Sequence Tags

From ChrX:

Let fetal DNA fraction be ϵ

	Maternal Contribution	Male Fetus Contribution	Female Fetus Contribution
ChrX	$2(1-\epsilon)$	ϵ	2ϵ

Male pregnancies ChrX sequence tag density (fetal and maternal)=$2(1-\epsilon)+\epsilon=2-\epsilon$

Female pregnancies ChrX sequence tag density (fetal and maternal)=$2(1-\epsilon)+2\epsilon=2$

Let x be the ratio of ChrX sequence tag density of male to female pregnancies. In this study, the denominator of this ratio is taken to be the median sequence tag density of all female pregnancies.

Thus, fetal DNA fraction $\epsilon=2(1-x)$

From ChrY:

Fetal DNA fraction ϵ=(sequence tag density of ChrY in maternal plasma/sequence tag density of ChrY in male plasma)

Note that in these derivations, we assume that the total number of sequence tags obtained is the same for all samples. In reality, the total number of sequence tags obtained for different sample is different, and we have taken into account such differences in our estimation of fetal DNA fraction by normalizing the sequence tag density of each chromosome to the median of the autosomal sequence tag densities for each sample.

Calculating Fetal DNA Fraction in Maternal Plasma of Aneuploid (Trisomy) Pregnancies:

Let fetal DNA fraction be ϵ

	Maternal Contribution	Trisomic Fetus Contribution	Disomic Fetus Contribution
Trisomic Chromosome	$2(1-\epsilon)$	3ϵ	2ϵ

$$\frac{\text{Trisomic pregnancies trisomic chromosome sequence counts(fetal and maternal)}}{\text{Disomic pregnancies trisomic chromosome sequence counts(fetal and maternal)}} = \frac{2(1-\varepsilon)+3\varepsilon}{2(1-\varepsilon)+2\varepsilon} = \frac{2+\varepsilon}{2}$$

Let x be the ratio of trisomic chromosome sequence counts (or sequence tag density) of trisomic to disomic pregnancies. In this study, the denominator of this ratio is taken to be the median sequence tag density of all disomic pregnancies.

Thus, fetal DNA fraction $\epsilon=2(x-1)$.

Example 8

Correction of Sequence Tag Density Bias Resulting from G/C or A/T Content Among Different Chromosomes in a Sample

This example shows a refinement of results indicating sequences mapping to different chromosomes and permitting the determination of the count of different chromosomes or regions thereof. That is, the results as shown in FIG. **1**A may be corrected to eliminate the variations in sequence tag density shown for chromosomes higher in G/C content, shown towards the right of the Figure. This spread of values results from sequencing bias in the method used, where a greater number of reads tend to be obtained depending on G/C content. The results of the method of this example are shown in FIG. **10**. FIG. **10** is an overlay which shows the results from a number of different samples, as indicated in the legend. The sequence tag density values in FIGS. **1** and **10** were normalized to those of a male genomic DNA control, since the density values are not always 1 for all the chromosomes (even after GC correction) but are consistent among a sample. For example, after GC correction, values from all samples for chr19 cluster around 0.8 (not shown). Adjusting the data to a nominal value of 1 can be done by plotting the value relative to the male gDNA control. This makes the values for all chromosomes cluster around 1

Outlying chromosome sequence tag densities can be seen as significantly above a median sequence tag density; disomic chromosomes are clustered about a line running along a density value of about 1. As can be seen there, the results from chromosome 19 (far right, highest in G/C content), for example, show a similar value when disomic as other disomic chromosomes. The variations between chromosomes with low and high G/C content are eliminated from the data to be examined. Samples (such as P13 in the present study) which could not have been unambiguously interpreted now may be. Since G/C content is the opposite of A/T content, the present method will correct for both. Either G/C bias or A/T bias can result from different sequencing methods. For example, it has been reported by others that the Solexa method results in a higher number of reads from sequences where the G/C content is high. See, Dohm et al., "Substantial biases in ultra-short read data sets from high-throughput DNA sequencing," *Nuc. Acids Res.* 36(16), e105; doi:10.1093/nar/gkn425. The procedure of the present example follows the following steps:

a. Calculate G/C content of the human genome. Calculate the G/C content of every 20 kb non-overlapping window of each chromosome of the human genome (HG18) using the hgG/CPercent script of the UCSC Genome Browser's "kent source tree," which contains different utility programs, available to the public under license. The output file contains the coordinate of each 20 kb bin and the corresponding G/C content. It was found that a large number of reads were obtained higher G/C ranges (about 55-70%) and very few reads were obtained at lower G/C content percentages, with essentially none below about 30% G/C (data not shown). Because the actual length of a sequenced DNA fragment is not known (we only sequenced the first 25 bp of one end of a piece of DNA on the flow cell), and it's the G/C content of the entire piece of DNA that contributed to sequencing bias, an arbitrary window of known human genomic DNA sequence is chosen for determining G/C content of different reads. We chose a 20 kb window to look at the relationship between number of reads and GC content. The window can be much smaller e.g., 10 kb or 5 kb, but a size of 20 kb makes computation easier.

b. Calculate the relationship between sequence coverage and G/C content. Assign weight to each read according to G/C content. For each sample, the number of read per 20 kb bin is counted. The number of read is plotted against G/C content. The average number of read is calculated for every 0.1% G/C content, ignoring bins with no reads, bins with zero G/C percent, and bins with over-abundant reads. The reciprocal of the average number of reads for a particular G/C percent relative to the global median number of read is calculated as the weight. Each read is then assigned a weight depending on the G/C percent of the 20 kb window it falls into.

c. Investigate the distribution of reads across each autosome and chromosome X. In this step, the number of reads, both unweighted and weighted, in each non-overlapping 50 kb window is recorded. For counting, we chose a 50 kb window in order to obtain a reasonable number of reads per window and reasonable number of windows per chromosome to look at the distributions. Window size may be selected based on the number of reads obtained in a given experiment, and may vary over a wide range. For example, 30K-100K may be used. Known microsatellite regions are ignored. A graph showing the results of chr1 of P7 is shown in FIG. **11**, which illustrates the weight distribution of this step (c) from sample P7, where the weight assigned to different G/C contents is shown; Reads with higher G/C content are overly represented than average and thus are given less weight.

d. Investigate the distribution of reads across chrY. Calculate the number of chrY reads in transcribed regions after applying weight to reads on chrY. Chromosome Y is treated

individually because it is short and has many repeats. Even female genome sequence data will map in some part to chromosome Y, due to sequencing and alignment errors. The number of chrY reads in transcribed regions after applying weight to reads on chrY is used to calculate percentage of fetal DNA in the sample.

Example 9

Comparing Different Patient Samples Using Statistical Analyses (t Statistic)

This example shows another refinement of results as obtained using the previous examples. In this case, multiple patient samples are analyzed in a single process. FIG. **12** illustrates the results of an analysis of patients P13, P19, P31, P23, P26, P40, P42, P1, P2, P6, P7, P14, P17, P20, P52, P53, P57, P59 and P64, with their respective karyotypes indicated, as in Table 1, above. The dotted line shows the 99% confidence interval, and outliers may be quickly identified. It may be seen by looking below the line that male fetuses have less chromosome X (solid triangles). An exception is P19, where it is believed that there were not enough total reads for this analysis. It may be seen by looking above the line that trisomy 21 patients (solid circles) are P1, 2, 6, 7, 14, 17, 20, 52 and 53. P57 and 59 have trisomy 18 (open diamonds) and P64 has trisomy 13 (star). This method may be presented by the following three step process:

Step 1: Calculate at statistic for each chromosome relative to all other chromosome in a sample. Each t statistic tells the value of each chromosome median relative to other chromosomes, taking into account the number of reads mapped to each chromosome (since the variation of the median scales with the number of reads). As described above, the present analyses yielded about 5 million reads per sample. Although one may obtain 3-10 million reads per sample, these are short reads, typically only about 20-100 bp, so one has actually only sequenced, for example about 300 million of the 3 billion by in the human genome. Thus, statistical methods are used where one has a small sample and the standard deviation of the population (3 billion, or 47 million for chromosome 21) is unknown and it is desired to estimate it from the sample number of reads in order to determine the significance of a numerical variation. One way to do this is by calculating Student's t-distribution, which may be used in place of a normal distribution expected from a larger sample. The t-statistic is the value obtained when the t-distribution is calculated. The formula used for this calculation is given below. Using the methods presented here, other t-tests can be used.

Step 2: Calculate the average t statistic matrix by averaging the values from all samples with disomic chromosomes. Each patient sample data is placed in at matrix, where the row is chr1 to chr22, and the column is also chr1 to chr22. Each cell represents the t value when comparing the chromosomes in the corresponding row and column (i.e., position (2,1) in the matrix is the t-value of when testing chr2 and chr1) the diagonal of the matrix is 0 and the matrix is symmetric. The number of reads mapping to a chromosome is compared individually to each of chr1-22.

Step 3: Subtract the average t statistic matrix from the t statistic matrix of each patient sample. For each chromosome, the median of the difference in t statistic is selected as the representative value.

The t statistic for 99% confidence for large number of samples is 3.09. Any chromosome with a representative t statistic outside −3.09 to 3.09 is determined as non-disomic.

Example 10

Calculation of Required Number of Sequence Reads after G/C Bias Correction

In this example, a method is presented that was used to calculate the minimum concentration of fetal DNA in a sample that would be needed to detect an aneuploidy, based on a certain number of reads obtained for that chromosome (except chromosome Y). FIG. **13** and FIG. **14** show results obtained from 19 patient plasma DNA samples, 1 donor plasma DNA sample, and duplicate runs of a donor gDNA sample. It is estimated in FIG. **13** that the minimum fetal DNA % of which over-representation of chr21 can be detected at the best sampling rate (~70 k reads mapped to chr21) is ~6%. (indicated by solid lines in FIG. **13**). The lines are drawn between about 0.7×10^5 reads and 6% fetal DNA concentration. It can be expected that higher numbers of reads (not exemplified here) the needed fetal DNA percentage will drop, probably to about 4%.

In FIG. **14**, the data from FIG. **13** are presented in a logarithmic scale. This shows that the minimum required fetal DNA concentration scales linearly with the number of reads in a square root relationship (slope of −0.5). These calculations were carried out as follows:

For large n (n>30), t statistic

$$t = \frac{\overline{y_2} - \overline{y_1}}{\sqrt{\frac{s_2^2}{n_2} + \frac{s_1^2}{n_1}}},$$

where $\overline{y_2}-\overline{y_1}$ is the difference in means (or amount of over- or under-representation of a particular chromosome) to be measured; s is the standard deviation of the number of reads per 50 kb in a particular chromosome; n is the number of samples (i.e., the number of 50 kb windows per chromosome). Since the number of 50 kb windows per chromosome is fixed, $n_1=n_2$. If we assume that

$$s_1 \approx s_2,$$

$$\overline{y_2} - \overline{y_1} \approx t\sqrt{\frac{2s_1^2}{n_1}}$$

$$= sqrt(2) * \text{half width of the confidence interval at confidence level governed by the value of } t.$$

Thus,

$$\frac{\overline{y_2}}{\overline{y_1}} - 1 \approx \frac{t\sqrt{\frac{2s_1^2}{n_1}}}{\overline{y_1}}.$$

For every chromosome in every sample, we can calculate the value

$$\frac{t\sqrt{\frac{2s_1^2}{n_1}}}{\overline{y_1}},$$

which corresponds to the minimum over- or under-representation

$$\left(\frac{\overline{y_2}}{\overline{y_1}}-1\right)$$

that can be resolved with confidence level governed by the value of t. Note that

$$2*\left(\frac{\overline{y_2}}{\overline{y_1}}-1\right)*100\%$$

corresponds to the minimum fetal DNA % of which any over- or under-representation of chromosomes can be detected. We expect the number of reads mapped to each chromosome to play a role in determining standard deviation s_1, since according to Poisson distribution, the standard deviation equals to the square root of the mean. By plotting

$$2*\left(\frac{\overline{y_2}}{\overline{y_1}}-1\right)*100\% \text{ vs.}$$

number of reads mapped to each chromosome in all the samples, we can evaluate the minimum fetal DNA % of which any over- or under-representation of chromosomes can be detected given the current sampling rate.

After correction of G/C bias, the number of reads per 50 kb window for all chromosomes (except chromosome Y) is normally distributed. However, we observed outliers in some chromosomes (e.g., a sub-region in chromosome 9 has near zero representation; a sub-region in chromosome 20 near the centromere has unusually high representation) that affect the calculation of standard deviation and the mean. We therefore chose to calculate confidence interval of the median instead of the mean to avoid the effect of outliers in the calculation of confidence interval. We do not expect the confidence interval of the median and the mean to be very different if the small number of outliers has been removed. The 99.9% confidence interval of the median for each chromosome is estimated from bootstrapping 5000 samples from the 50 kb read distribution data using the percentile method. The half width of the confidence interval is estimated as 0.5*confidence interval. We plot 2*(half width of confidence interval of median)/median*100% vs. number of reads mapped to each chromosome for all samples.

Bootstrap resampling and other computer-implemented calculations described here were carried out in MATLAB®, available from The Mathworks, Natick, Mass.

CONCLUSION

The above specific description is meant to exemplify and illustrate the invention and should not be seen as limiting the scope of the invention, which is defined by the literal and equivalent scope of the appended claims. Any patents or publications mentioned in this specification are intended to convey details of methods and materials useful in carrying out certain aspects of the invention which may not be explicitly set out but which would be understood by workers in the field. Such patents or publications are hereby incorporated by reference to the same extent as if each was specifically and individually incorporated by reference, as needed for the purpose of describing and enabling the method or material referred to.

REFERENCES

1. Cunningham F, et al. (2002) in Williams Obstetrics (McGraw-Hill Professional, New York), p. 942.
2. (2007) ACOG Practice Bulletin No. 88, December 2007. Invasive prenatal testing for aneuploidy. *Obstet Gynecol,* 110: 1459-1467.
3. Wapner R, et al. (2003) First-trimester screening for trisomies 21 and 18. *N Engl J Med,* 349: 1405-1413.
4. Alfirevic Z, Neilson J P (2004) Antenatal screening for Down's syndrome. *Bmj* 329: 811-812.
5. Malone F D, et al. (2005) First-trimester or second-trimester screening, or both, for Down's syndrome. *N Engl J Med,* 353: 2001-2011.
6. Herzenberg L A, et al. (1979) Fetal cells in the blood of pregnant women: detection and enrichment by fluorescence-activated cell sorting. *Proc Natl Acad Sci USA,* 76: 1453-1455.
7. Bianchi D W, et al. (1990) Isolation of fetal DNA from nucleated erythrocytes in maternal blood. *Proc Natl Acad Sci USA,* 87: 3279-3283.
8. Cheung M C, Goldberg J D, Kan Y W (1996) Prenatal diagnosis of sickle cell anaemia and thalassaemia by analysis of fetal cells in maternal blood. *Nat Genet,* 14: 264-268.
9. Bianchi D W, et al. (1997) PCR quantitation of fetal cells in maternal blood in normal and aneuploid pregnancies. *Am J Hum Genet,* 61: 822-829.
10. Bianchi D W, et al. (2002) Fetal gender and aneuploidy detection using fetal cells in maternal blood: analysis of NIFTY I data. National Institute of Child Health and Development Fetal Cell Isolation Study. *Prenat Diagn,* 22: 609-615.
11. Lo Y M, et al. (1997) Presence of fetal DNA in maternal plasma and serum. *Lancet,* 350: 485-487.
12. Dennis Lo Y M, Chiu R W (2007) Prenatal diagnosis: progress through plasma nucleic acids. *Nat Rev Genet,* 8: 71-77.
13. Lo Y M, et al. (1998) Quantitative analysis of fetal DNA in maternal plasma and serum: implications for noninvasive prenatal diagnosis. *Am J Hum Genet,* 62: 768-775.
14. Lo Y M, et al. (2007) Plasma placental RNA allelic ratio permits noninvasive prenatal chromosomal aneuploidy detection. *Nat Med,* 13: 218-223.
15. Tong Y K, et al. (2006) Noninvasive prenatal detection of fetal trisomy 18 by epigenetic allelic ratio analysis in maternal plasma: Theoretical and empirical considerations. *Clin Chem,* 52: 2194-2202.
16. Dhallan R, et al. (2007) A non-invasive test for prenatal diagnosis based on fetal DNA present in maternal blood: a preliminary study. *Lancet,* 369: 474-481.
17. Fan H C, Quake S R (2007) Detection of aneuploidy with digital polymerase chain reaction. *Anal Chem,* 79: 7576-7579.
18. Lo Y M, et al. (2007) Digital PCR for the molecular detection of fetal chromosomal aneuploidy. *Proc Natl Acad Sci USA,* 104: 13116-13121.
19. Quake S R, Fan H C. (2006). Non-invasive fetal genetic screening by digital analysis. USA Provisional Patent

Application No. 60/764,420. 20. Mardis E R (2008) Next-Generation DNA Sequencing Methods. *Annu Rev Genomics Hum Genet,* 9: 387-402.

20. Lander E S, et al. (2001) Initial sequencing and analysis of the human genome. *Nature,* 409: 860-921.
21. Chan K C, et al. (2004) Size distributions of maternal and fetal DNA in maternal plasma. *Clin Chem,* 50: 88-92.
22. Li Y, et al. (2004) Size separation of circulatory DNA in maternal plasma permits ready detection of fetal DNA polymorphisms. *Clin Chem,* 50: 1002-1011.
23. Cooper G, Hausman R (2007) in The cell: a molecular approach (Sinauer Associates, Inc, Sunderland), p. 168.
24. Jahr S, et al. (2001) DNA fragments in the blood plasma of cancer patients: quantitations and evidence for their origin from apoptotic and necrotic cells. *Cancer Res,* 61: 1659-1665.
25. Giacona M B, et al. (1998) Cell-free DNA in human blood plasma: length measurements in patients with pancreatic cancer and healthy controls. *Pancreas,* 17: 89-97.
26. Schones D E, et al. (2008) Dynamic regulation of nucleosome positioning in the human genome. *Cell,* 132: 887-898.
27. Ozsolak F, Song J S, Liu X S, Fisher D E (2007) High-throughput mapping of the chromatin structure of human promoters. *Nat Biotechnol,* 25: 244-248.
28. Yuan G C, et al. (2005) Genome-scale identification of nucleosome positions in S. cerevisiae. *Science,* 309: 626-630.
29. Lee W, et al. (2007) A high-resolution atlas of nucleosome occupancy in yeast. *Nat Genet,* 39: 1235-1244.
30. Sohda S, et al. (1997) The proportion of fetal nucleated red blood cells in maternal blood: estimation by FACS analysis. *Prenat Diagn,* 17: 743-752.
31. Hamada H, et al. (1993) Fetal nucleated cells in maternal peripheral blood: frequency and relationship to gestational age. *Hum Genet,* 91: 427-432.
32. Nelson J L (2008) Your cells are my cells. *Sci Am,* 298: 64-71.
33. Khosrotehrani K, Bianchi D W (2003) Fetal cell microchimerism: helpful or harmful to the parous woman? *Curr Opin Obstet Gynecol,* 15: 195-199.
34. Lo Y M, et al. (1999) Rapid clearance of fetal DNA from maternal plasma. *Am J Hum Genet,* 64: 218-224.
35. Smid M, et al. (2003) No evidence of fetal DNA persistence in maternal plasma after pregnancy. *Hum Genet,* 112: 617-618.
36. Rijnders R J, Christiaens G C, Soussan A A, van der Schoot C E (2004) Cell-free fetal DNA is not present in plasma of nonpregnant mothers. *Clin Chem,* 50: 679-681; author reply 681.
37. Hillier L W, et al. (2008) Whole-genome sequencing and variant discovery in *C. elegans. Nat Methods,* 5: 183-188.
38. Dohm J C, Lottaz C, Borodina T, Himmelbauer H (2008) Substantial biases in ultra-short read data sets from high-throughput DNA sequencing. *Nucleic Acids Res.*
39. Harris T D, et al. (2008) Single-molecule DNA sequencing of a viral genome. *Science,* 320: 106-109.
40. Samura O, et al. (2003) Cell-free fetal DNA in maternal circulation after amniocentesis. *Clin Chem,* 49: 1193-1195.
41. Lo Y M, et al. (1999) Increased fetal DNA concentrations in the plasma of pregnant women carrying fetuses with trisomy 21. *Clin Chem,* 45: 1747-1751.
42. Segal E, et al. (2006) A genomic code for nucleosome positioning. *Nature,* 442: 772-778.

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What is claimed is:

1. A method of testing for an abnormal distribution of a chromosome in a sample comprising a mixture of maternal and fetal DNA, comprising the steps of:

(a) obtaining maternal and fetal DNA from said sample;

(b) sequencing predefined subsequences of the maternal and fetal DNA to obtain a plurality of sequence tags aligning to the predefined subsequences, wherein said sequence tags are of sufficient length to be assigned to a specific predefined subsequence, wherein the predefined subsequences are from a plurality of different chromosomes, and wherein said plurality of different chromosomes comprise at least one first chromosome suspected of having an abnormal distribution in said sample and at least one second chromosome presumed to be normally distributed in said sample;

(c) assigning the plurality of sequence tags to their corresponding predetermined subsequences;

(d) determining a number of sequence tags aligning to the predetermined subsequences of said first chromosome and a number of sequence tags to the predetermined subsequences of the second chromosome; and

(e) comparing the numbers from step (d) to determine the presence or absence of an abnormal distribution of said first chromosome.

2. The method of claim 1 wherein the sample is a maternal serum or plasma sample, wherein the abnormal distribution of said first chromosome is a fetal aneuploidy, and wherein said second chromosome is a euploid chromosome.

3. The method of claim 2 wherein the sequencing comprises massively parallel sequencing of the predefined subsequences.

4. The method of claim 3 wherein said massively parallel sequencing comprises attaching DNA fragments to an optically transparent surface, conducing solid phase amplification of the attached DNA fragments to create a high density sequencing flow cell with millions of DNA clusters, and sequencing the DNA clusters by a four-color DNA sequencing-by-synthesis method employing reversible terminators with removable fluorescent dyes.

5. The method of claim 2 wherein the fetal aneuploidy is an aneuploidy of a chromosome selected from the group consisting of chromosome 13, chromosome 18 and chromosome 21.

6. The method of claim 2 wherein the step of assigning sequence tags to corresponding chromosome portions allows one mismatch.

7. The method of claim 2 wherein the length of the sequence tags is from about 25 bp to about 100 bp in length.

8. The method of claim 2 wherein the DNA is genomic DNA.

9. The method of claim 2 wherein said sequencing comprises selectively sequencing nucleic acid molecules comprising the predefined sequences.

10. The method of claim 9 wherein said sequencing comprises the use of a sequencing array.

11. The method of claim 10 wherein said selected defined subsequences of the genomic DNA are rendered single-stranded and captured under hybridizing conditions by single-stranded probes physically separated on an array.

12. The method of claim 2 further comprising determination of fetal DNA fraction of the DNA obtained from the maternal serum or plasma sample.

13. The method of claim 12 wherein the fetal DNA fraction is determined by digital PCR.

14. A method of testing for an abnormal distribution of a chromosome in a sample comprising a mixture of maternal and fetal DNA, comprising the steps of:

(a) obtaining maternal and fetal DNA from said sample;

(b) sequencing predefined subsequences of the maternal and fetal DNA to obtain a plurality of sequence tags aligning to the predefined subsequences, wherein said sequence tags are of sufficient length to be assigned to a specific predefined subsequence, wherein the predefined subsequences are from a plurality of different chromosomes, and wherein said plurality of different chromosomes comprise at least one first chromosome suspected of having an abnormal distribution in said sample and at least one second chromosome presumed to be normally distributed in said sample;

(c) assigning the plurality of sequence tags to their corresponding predetermined subsequences;

(d) determining a relative number of sequence tags aligning to the predetermined subsequences of said first chromosome and to the predetermined subsequences of said second chromosome;

(e) determining a weight for correcting for G/C bias and applying the weight to the numbers of sequence tags determined in step (d) to obtain a corrected number of sequence tags assigned to the predefined subsequences of the first chromosome and a corrected number of sequence tags assigned to the predefined subsequences of the second chromosome; and

(f) comparing the corrected number of sequence tags aligning to the predetermined subsequences of said first chromosome to the corrected number of sequence tags aligning to the predetermined subsequences of said second chromosome to determine the presence or absence of an abnormal distribution of said first chromosome.

15. The method of claim 14 wherein the sample is a maternal serum or plasma sample, wherein the abnormal distribution of said first chromosome is a fetal aneuploidy, and wherein said second chromosome is a euploid chromosome.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 8,296,076 B2
APPLICATION NO. : 13/452083
DATED : October 23, 2012
INVENTOR(S) : Hei-Mun Christina Fan and Stephen R. Quake

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Title Page, Item (54) in the title, line 2, replace "ANEUOPLOIDY" with --ANEUPLOIDY--.

Signed and Sealed this
Twenty-fifth Day of December, 2012

David J. Kappos

David J. Kappos
Director of the United States Patent and Trademark Office

UNITED STATES PATENT AND TRADEMARK OFFICE

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PATENT NO. : 8,296,076 B2
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Title Page, Item (54), line 2, and at Column 1, line 2, in the title, replace "ANEUOPLOIDY" with --ANEUPLOIDY--.

This certificate supersedes the Certificate of Correction issued December 25, 2012.

Signed and Sealed this
Nineteenth Day of February, 2013

Teresa Stanek Rea
Acting Director of the United States Patent and Trademark Office

CERTIFICATE OF SERVICE

I hereby certify that on this 4th day of May, 2015, the foregoing OPENING BRIEF OF APPELLANT was filed electronically with the U.S. Court of Appeals for the Federal Circuit by means of the Court's CM/ECF system. I further certify that the foregoing was served by means of electronic mail, as well as by the Court's CM/ECF system, which should have sent a Notice of Docket Activity, upon the following counsel of record for Appellee:

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CERTIFICATE OF COMPLIANCE

This brief complies with the type-volume limitation of Federal Rule of Appellate Procedure 32(a)(7)(B) because this brief contains 11,202 words, excluding the parts of the brief exempted by Federal Rule of Appellate Procedure 32(a)(7)(B)(iii).

This brief complies with the typeface requirements of Federal Rule of Appellate Procedure 32(a)(5) and the type-style requirements of Federal Rule of Appellate Procedure 32(a)(6) because this brief has been prepared in proportionally spaced typeface using Microsoft Word in 14-point Times New Roman font.

Dated: May 4, 2015

Respectfully submitted,

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